Gene Expression of Insulin Signal-Transduction Pathway Intermediates Is Lower in Rats Fed a Beef Tallow Diet Than in Rats Fed a Safflower Oil Diet

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To elucidate the effects of dietary fatty acid composition on the insulin signaling pathway, we measured the gene expression of the earliest steps in the insulin action pathway in skeletal muscle of rats fed a safflower oil diet or a beef tallow diet. Rats were meal-fed an isoenergetic diet based on either safflower oil or beef tallow for 8 weeks. Both diets provided 45%, 35%, and 20% of energy as fat, carbohydrate, and protein, respectively. Insulin resistance, assessed from the diurnal rhythm of plasma glucose and insulin and the oral glucose tolerance test (OGTT), developed in rats fed a beef tallow diet. Body fat content was greater in rats fed a beef tallow diet versus a safflower oil diet. The level of insulin receptor mRNA, relative expression of the insulin receptor mRNA isoforms, and receptor protein were not affected by the composition of dietary fatty acids. The abundance of insulin receptor substrate-1 (IRS-1) and phosphatidylinositol (PI) 3-kinase mRNA and protein was significantly lower in rats fed a beef tallow diet versus a safflower oil diet. We conclude that long-term feeding of a high-fat diet with saturated fatty acids induces decrease in IRS-1 and PI 3-kinase mRNA and protein levels, causing insulin resistance in skeletal muscle.

THE INCREASING RECOGNITION of obesity, insulin resistance, and hyperinsulinemia as a risk factor for cardiovascular disease has highlighted the search for environmental promoters of these syndromes.[1] In addition to dietary fat consumption, epidemiological studies have shown an association between the fatty acid composition of the diet and the incidence of obesity, diabetes, and coronary heart disease.[2-6] Consumption of a diet high in saturated fatty acids induced obesity and insulin resistance.[7-9] We recently reported that when rats were meal-fed an isoenergetic diet (45% of energy as fat) based on beef tallow (rich in saturated and monounsaturated fatty acids) or safflower oil (rich in n-6 polyunsaturated fatty acids) for 8 to 16 weeks, body fat accumulation was greater in rats fed a beef tallow diet than in those fed a safflower oil diet.[10,11] The clinical severity of the insulin resistance and the effect of insulin on daily glucose homeostasis depend on the composition of dietary fatty acids,[12-15] although the mechanism on a molecular basis remains unknown.

Insulin initiates the biological effects by binding to its cell surface receptor on target tissues. Activation of insulin receptor tyrosine kinase, subsequent phosphorylation of the insulin receptor β-subunit and the insulin receptor substrate-1 (IRS-1), and phosphatidylinositol (PI) 3-kinase are thought to be essential steps in insulin stimulation of glucose metabolism. Thus, the insulin receptor, IRS-1, and PI 3-kinase represent three of the earliest steps in insulin action at the cellular level.[16,17] Insulin resistance might be caused by abnormalities in insulin signal-transduction pathways at any level of the signal-transmitting chain in the target cell. Therefore, the present study was designed to examine gene expression of insulin signal-transduction pathway intermediates in rats fed a safflower oil diet or a beef tallow diet.

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MATERIALS AND METHODS

Animals and Diets

Forty male Sprague-Dawley rats (5 weeks old) were obtained from CLEA Japan (Tokyo). Half of the animals were fed a safflower oil diet and the other half a beef tallow diet. The composition of both diets has been described previously.[10] Both diets provided 45%, 35%, and 20% of energy as fat, carbohydrate, and protein, respectively. The metabolizable energy was 19.7 kJ/g for the safflower oil diet and 18.4 kJ/g for the beef tallow diet. The fatty acid composition of safflower oil and beef tallow has been described previously[11]; safflower oil consisted of 79% linoleic acid, and beef tallow consisted of 44% oleic, 27% palmitic, and 18% stearic acids.

Experimental Design

The animals were individually caged at 23°C with light from 7:00 AM to 7:00 PM. Each group was meal-fed the diet at 8:00 to 9:00 AM and 8:00 to 9:00 PM and allowed free access to water for 8 weeks. Both groups of rats were offered the appropriate diet in an amount such that the two groups consumed equal metabolizable energy during the experimental period. The amount of diet fed to the rats was increased gradually from 180 kJ/d for the first week to 326 kJ/d for the eighth week of the experiment. On the final day of the experiment, each group was killed by decapitation after an overnight fast. The gastrocnemius muscle and abdominal adipose tissues were removed and weighed, and then stored at −80°C until use. All procedures involving animals were approved by the experimental animal care committee of the University of Tsukuba.

Diurnal Rhythm

Diurnal variations of plasma glucose and insulin were measured at 2:30, 6:30, and 10:30 AM and 2:30, 6:30, and 10:30 PM in rats after 4 weeks of feeding the experimental diet. For determination of

Table 1. Sequences of PCR Primers

Gene	Primer	Nucleotide No.	Size of PCR Product (bp)	GenBank Accession No.
Insulin receptor	5′-CCTGATAACTGTCCAGAGAG-3′	4122-4141	289	M29014
	5′-TCCGTTTGATGCTCAGAGAG-3′	4410-4391		
Insulin receptor isoforms (exon 11+, exon 11−)	5′-GTGCTGCTCATGTCCTAAGA-3′	2474-2493	270, 234	M29014
	5′-AATGGTCTGTGCTCTTCGTG-3′	2743-2724		
IRS-1	5′-GCCAATCTTCATCCAGTTGC-3′	1382-1401	337	X58375
	5′-CATCGTGAAGAAGGCATAGG-3′	1718-1699		
PI 3-kinase	5′-CAGGATCAAGTTGTCAAAGAAGAT-3′	1297-1320	231	M60651
	5′-TATGTATTCTTTGCTGTACCGCTC-3′	1527-1504		
β-Actin	5′-CCTAGCACCATGAAGATCAA-3′	2846-2865	377	J00691
	5′-AGCCATGCCAAATGTCTCAT-3′	3222-3203		

NOTE. Sequences of each primer pair and their location in sequences cited in the GenBank database are presented.

plasma insulin and glucose, a small incision was made in the tail artery within 1 minute of removing the animal from its cage, and blood samples (150 μL) were collected into tubes coated with heparin and NaF. Plasma samples were stored at −80°C until analysis.

Oral Glucose Tolerance Test

At age 10 weeks, an oral glucose tolerance test (OGTT) was performed after an overnight fast. A solution of 2 glucose/kg body weight was administered orally.[18] Blood samples were collected for

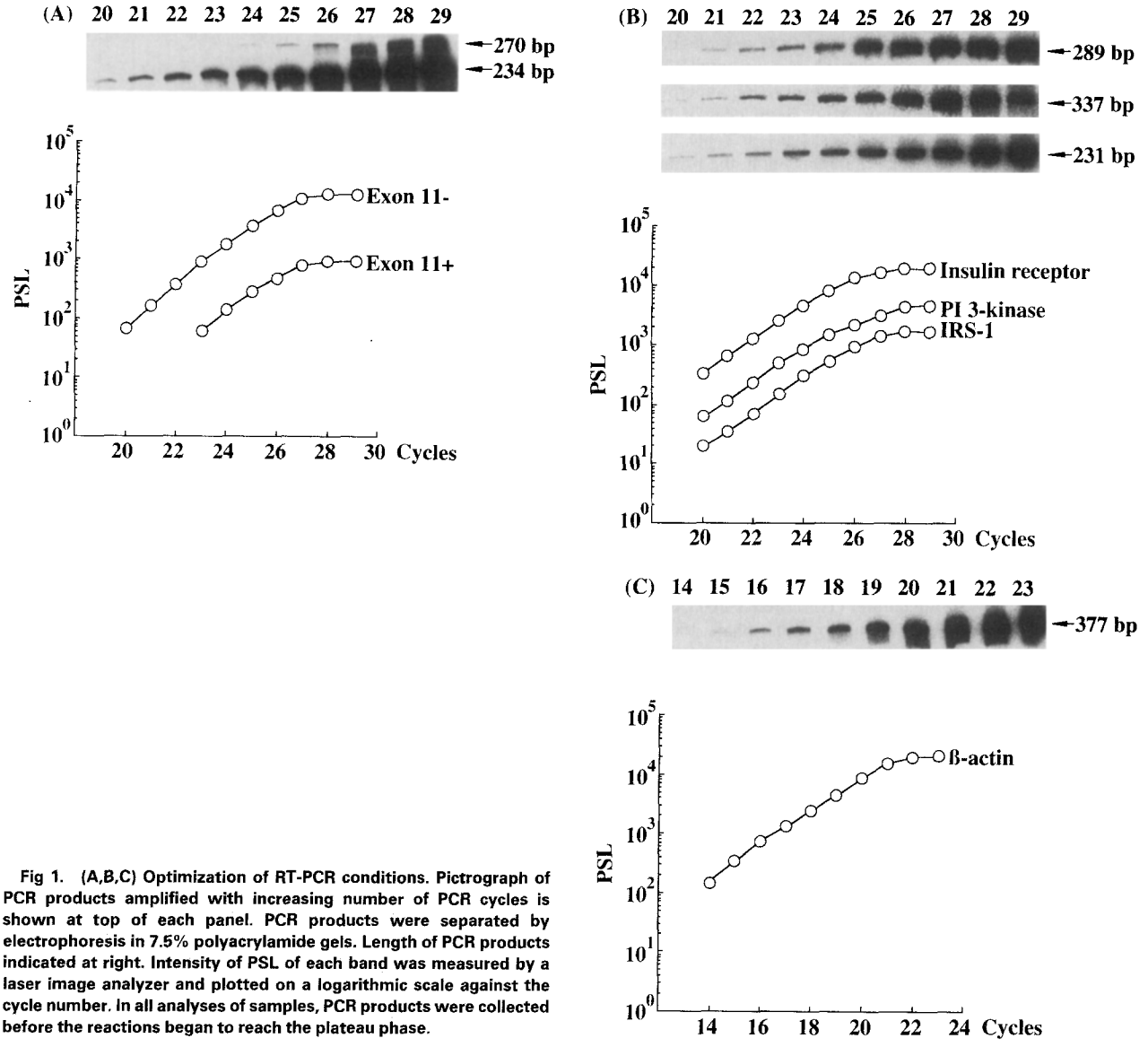

Fig 1. (A,B,C) Optimization of RT-PCR conditions. Pictograph of PCR products amplified with increasing number of PCR cycles is shown at top of each panel. PCR products were separated by electrophoresis in 7.5% polyacrylamide gels. Length of PCR products indicated at right. Intensity of PSL of each band was measured by a laser image analyzer and plotted on a logarithmic scale against the cycle number. In all analyses of samples, PCR products were collected before the reactions began to reach the plateau phase.

Table 2. Characteristics of Rats Fed a Safflower Oil or Beef Tallow Diet

Characteristic	Safflower Oil (n = 10)	Beef Tallow (n = 10)
Body weight (g)		
Initial	139 ± 1	136 ± 1
Final	424 ± 4	422 ± 4
Gain	285 ± 4	286 ± 4
Body fat (g)	58 ± 2	75 ± 3*
Gastrocnemius muscle		
Weight (g)	5.56 ± 0.14	5.39 ± 0.15
RNA (μg/g tissue)	887 ± 23	902 ± 19
Protein (mg/g tissue)	144 ± 7	135 ± 7

NOTE. Data are the mean ± SE.
*$P < .01$ *v* safflower oil group.

determination of plasma insulin and glucose at 0, 15, 30, 60, and 120 minutes.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from skeletal muscle with a guanidium thiocyanate water-saturated phenol extraction method.[19] First-strand cDNA synthesis was performed on 5 μg total RNA using oligo(dT) as described by the manufacturer (BRL SuperScript Kit; Life Technologies, Gaithersburg, MD). Sequences of the primers[20-23] used for amplification are shown in Table 1. Before polymerase chain reaction (PCR) amplification, the primers were labeled with [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) by using T4 polynucleotide kinase (Takara, Kyoto, Japan). The reactions were performed in a DNA Thermal Cycler (Perkin-Elmer, Branchburg, NJ) using the following cycle conditions: initial denaturation at 94°C for 6 minutes, denaturation at 94°C for 1 minute, annealing at 58°C for 2 minutes (at 62°C in the case of PI 3-kinase), and extension at 72°C for 3 minutes.[24] Optimization of reverse transcriptase (RT) PCR conditions is shown in Fig 1. PCR reactions were repeated 25 cycles for the insulin receptor, IRS-1, and PI 3-kinase and 18 cycles for β-actin within the exponential and linear range. PCR products (10 μL) were analyzed by electrophoresis in 7.5% polyacrylamide gels. The amount of mRNA in each sample was quantified by a laser image analyzer (Fujix BAS2000; Fuji Film, Tokyo, Japan).

Western Blotting Analysis

The frozen samples of gastrocnemius muscle were homogenized by a polytron in solubilization buffer (1% Triton X-100, 50 mmol/L HEPES (pH 7.4), 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L phenylmethylsulfonyl fluoride, and 0.1 mg/mL aprotinin) and centrifuged at 55,000 rpm at 4°C for 60 minutes, and the supernatant was collected. Equal amounts of protein (300 μg) were size-fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5% or 10% Tris acrylamid) according to the method of Laemmli,[25] and transferred onto Immobilon polyvinylidene fluoride membrane (Millipore) as described by Towbin et al.[26] Nonspecific protein binding to the membrane was reduced by preincubating the membrane overnight at 4°C in blocking buffer (3% bovine serum albumin, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02% Tween 20). The membranes were then incubated with anti–insulin receptor β (Santa Cruz Biotechnology, Santa Cruz, CA), anti–IRS-1, and anti–PI3-kinase (Upstate Biotechnology) antibody for 4 hours at 22°C and washed for 60 minutes with the blocking buffer without BSA.[27] Bound antibodies were detected by incubation with ^{125}I-protein A (Amersham) for 1 hour at 22°C. Band intensities were quantified by a laser image analyzer (Fujix BAS2000).

Cloning and Sequencing of Rat PI 3-Kinase cDNA

A 231-bp PCR fragment of rat PI 3-kinase cDNA was obtained using oligonucleotide primers (Table 1) based on the nucleotide sequences of mouse PI 3-kinase cDNA.[22] DNA sequencing was performed by a chain-termination method using Taq Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer) and a 373A DNA sequencer (Perkin Elmer).

Assays

Plasma insulin concentration was determined by enzyme immunoassay (Sanko-Junyaku, Japan), and glucose was analyzed enzymatically (Wako Pure Chemical Industries, Japan). Carcass fat was analyzed by the method of Mickelsen and Anderson.[28]

Statistical Analysis

The data are expressed as the mean ± SE. Statistical analysis of the diurnal rhythm of plasma insulin and glucose was performed

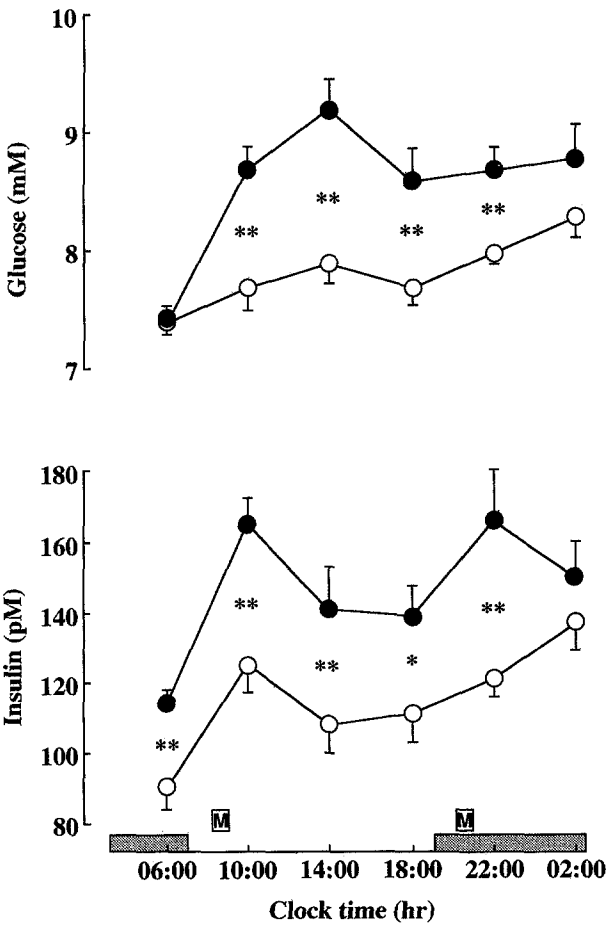

Fig 2. Diurnal variation of plasma glucose and insulin in rats fed a safflower oil diet (○) or a beef tallow diet (●) after 4 weeks of feeding. Data are the mean ± SE for 9 to 10 rats per group. *$P < .05$, **$P < .01$: *v* safflower oil group. M, meal; ▨, darkness.

using two-way ANOVA. Where ANOVA yielded significant intergroup differences, the data were further analyzed by Student's *t* test. All other data were assessed by Student's *t* test.

RESULTS

Basic Profiles

Both groups of rats had the same body weight gain during the 8-week experimental period (Table 2). Body fat weight (sum of the lipid of carcass and abdominal adipose tissue) was significantly higher in rats fed a beef tallow diet than in those fed a safflower oil diet (Table 2). These results were similar to those reported previously.[10,11]

After feeding the experimental diets for 4 weeks, the diurnal variation of plasma glucose and insulin was measured. Plasma glucose and insulin levels were higher in almost all the tests during the day in rats fed a beef tallow diet than in those fed a safflower oil diet (Fig 2). Plasma glucose and insulin concentrations during the OGTT are illustrated in Fig 3. Plasma insulin was significantly increased but glucose was similar in the beef tallow diet group compared with the safflower oil diet group.

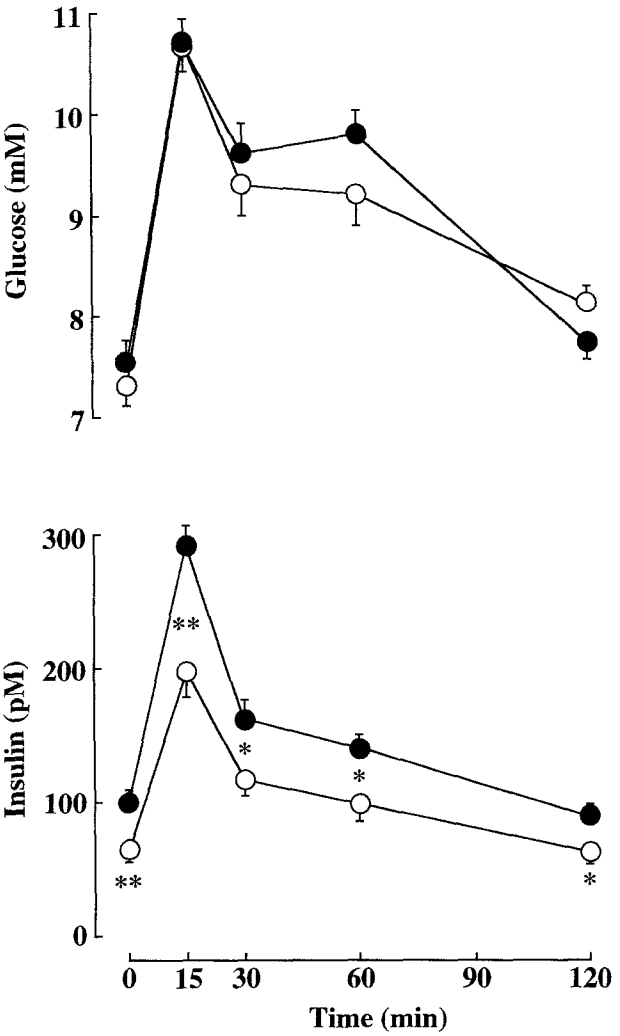

Fig 3. Plasma glucose and insulin responses to OGTT in rats fed a safflower oil diet (○) or beef tallow diet (●) after 5 weeks of feeding. Data are the mean ± SE for 9 to 10 rats per group. *$P < .05$, **$P < .01$: *v* safflower oil group.

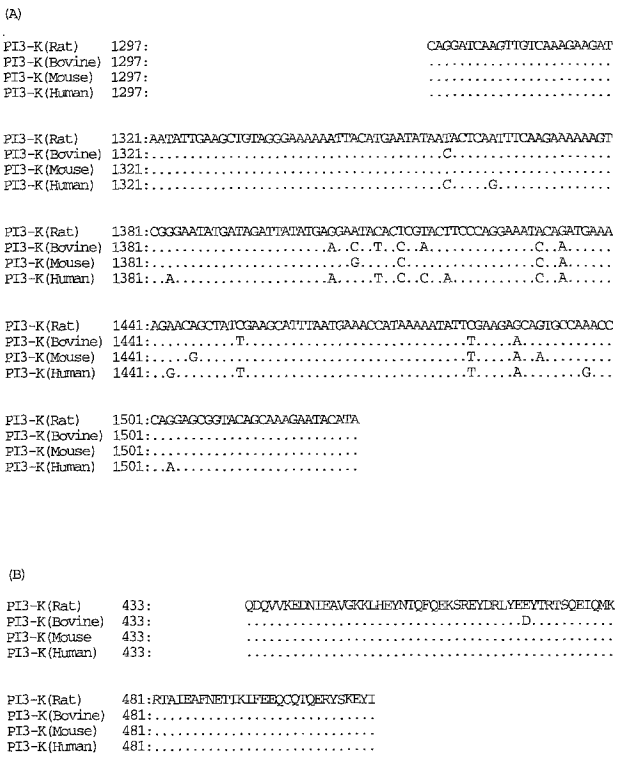

Fig 4. Comparison of nucleotide (A) and deduced amino acid (B) sequences of a rat PI 3-kinase cDNA to those of other species. Dots indicate nucleotide and amino acid identity with rat. The nucleotide sequence data reported herein will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D63325.

PI 3-Kinase cDNA

To assay PI 3-kinase mRNA in rats fed a safflower oil diet and a beef tallow diet, we cloned rat PI 3-kinase cDNA using the RT-PCR technique. Rat PI 3-kinase cDNA is similar to those of bovine (95.2% nucleotide and 98.7% amino acid identity),[29] mouse (96.5% nucleotide and 100% amino acid identity),[22] and human (93.1% nucleotide and 100% amino acid identity) origin[30] (Fig 4).

Gene Expression of Insulin-Signaling Pathway Intermediates

The wet weight, total RNA, and protein content of gastrocnemius muscle were not different between the two groups (Table 2). The level of insulin receptor mRNA was not affected by the composition of dietary fatty acids (Fig 5). Two specific PCR products were present, showing the two insulin receptor mRNA isoforms. Exon 11- was the predominant isoform in skeletal muscle (Fig 6). The relative expression of insulin receptor mRNA isoforms was not different between the two groups (Fig 6). The mRNA level of IRS-1, a principal substrate of the insulin receptor tyrosine kinase, was determined and was significantly lower

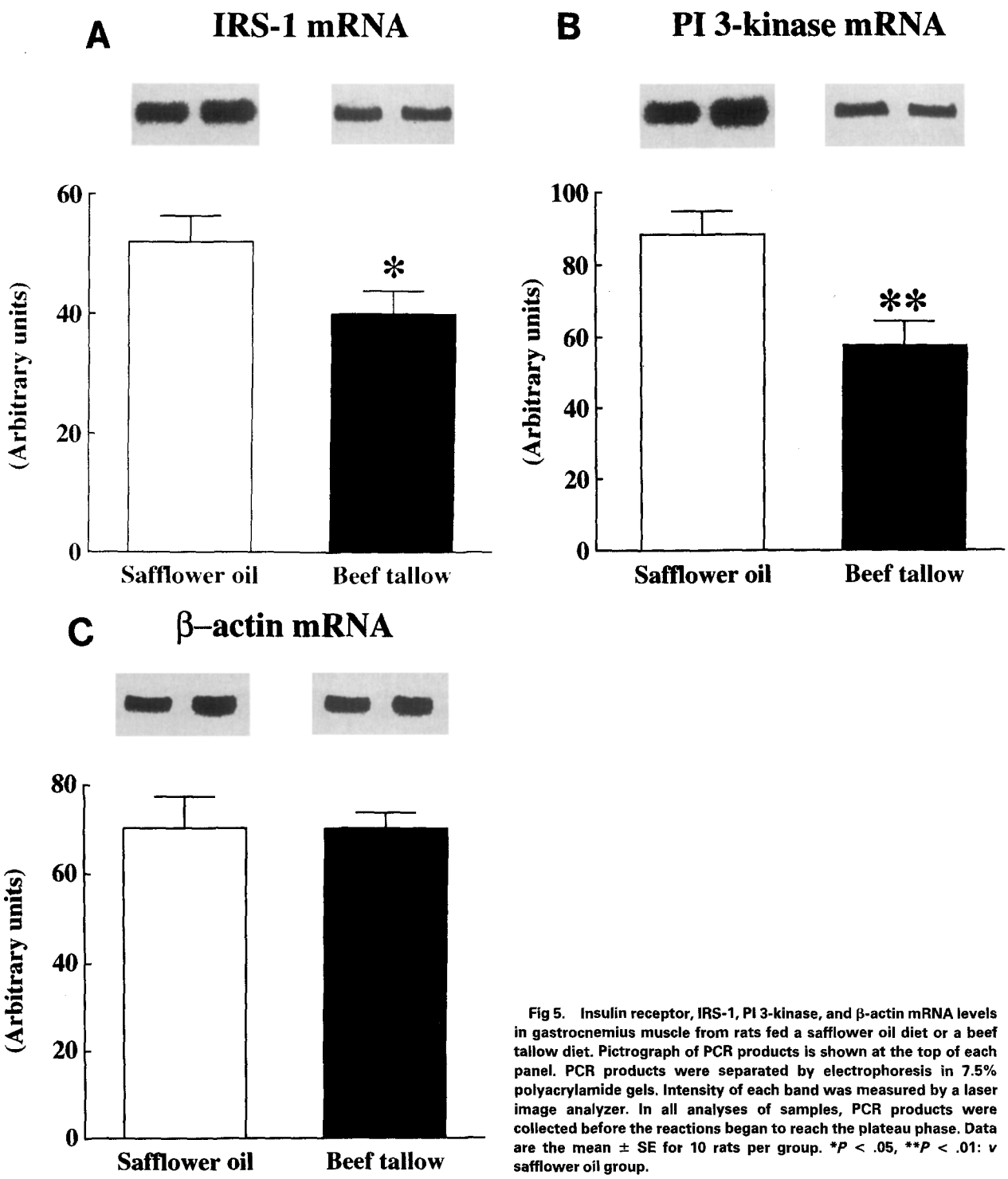

Fig 5. Insulin receptor, IRS-1, PI 3-kinase, and β-actin mRNA levels in gastrocnemius muscle from rats fed a safflower oil diet or a beef tallow diet. Pictrograph of PCR products is shown at the top of each panel. PCR products were separated by electrophoresis in 7.5% polyacrylamide gels. Intensity of each band was measured by a laser image analyzer. In all analyses of samples, PCR products were collected before the reactions began to reach the plateau phase. Data are the mean ± SE for 10 rats per group. *$P < .05$, **$P < .01$: *v* safflower oil group.

in rats fed a beef tallow diet than in those fed a safflower oil diet (Fig 5). The PI 3-kinase mRNA level was also significantly lower in the beef tallow diet group versus the safflower oil diet group (Fig 5). There was no significant difference in the level of β-actin mRNA in skeletal muscle between the two groups (Fig 5). We also measured protein levels of insulin receptor, IRS-1, and PI 3-kinase using Western blotting. The abundance of insulin receptor protein was not altered, but IRS-1 and PI 3-kinase protein were significantly lower in the beef tallow diet group than in the safflower oil diet group (Fig 7).

DISCUSSION

Rats fed a diet high in saturated fatty acids (beef tallow) compared with rats fed a diet high in unsaturated fatty acids (safflower oil) exhibit insulin resistance and hyperinsulin-

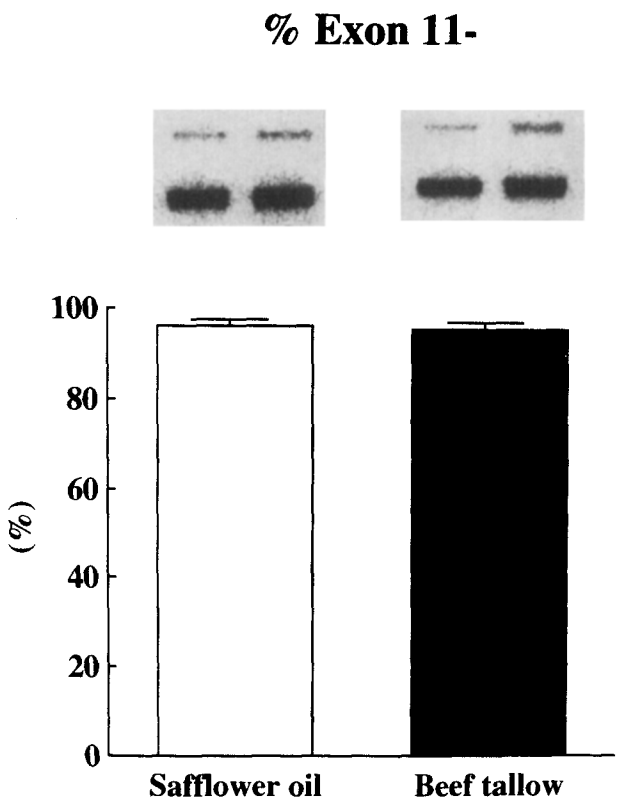

Fig 6. Relative expression of exon 11- in gastrocnemius muscle from rats fed a safflower oil diet or a beef tallow diet. Pictrograph of PCR products is shown at top. PCR products were separated by electrophoresis in 7.5% polyacrylamide gels. Upper band indicates exon 11+ (270 bp), and lower band indicates exon 11- (234 bp). Intensity of each band was measured by a laser image analyzer. In all analyses of samples, PCR products were collected before the reactions began to reach the plateau phase. Data are the mean ± SE for 10 rats per group.

emia throughout a full diurnal cycle and after a oral glucose load. To elucidate the underlying molecular mechanism of the effect of dietary fatty acid composition on the development of insulin resistance,[8,9] we examined the gene expression of insulin signal-transduction intermediates in skeletal muscle of rats fed a safflower oil diet or a beef tallow diet.

The decrease in insulin receptor concentration and number in skeletal muscle was observed in hypothalamic and genetically obese (ob/ob) mice.[31,32] On the other hand, insulin binding to skeletal muscle in other types of genetically obese (db/db) mice without hyperinsulinemia was normal.[32] Thus, there is an inverse relationship between the number of insulin receptors in target tissues and the concentration of insulin in blood,[31,33-35] suggesting that varying extents of the decrease in insulin binding reflect degrees of hyperinsulinemia. These observations are most likely a consequence of hyperinsulinemia, which results in downregulation of cell surface receptors.[36,37] In the present study, insulin receptor mRNA abundance was almost the same, but protein levels tended to be lower in the beef tallow diet group compared with the safflower oil diet group. Insulin receptor exists in two isoforms, which are generated by alternative splicing of a primary gene transcript and differ by a 12–amino acid insertion sequence corresponding to exon 11. The two isoforms possess distinct functional properties and are expressed in a tissue-specific fashion.[20,38-41] A recent report showed that non–insulin-dependent diabetes mellitus (NIDDM) with hyperinsulinemia is associated with an increase in an insulin receptor mRNA isoform without exon 11.[42] In contrast, in diabetes without severe hyperinsulinemia, the relative expression of insulin receptor isoforms was similar to that of nondiabetic controls.[43] Differences in the relative expression of the two receptor isoforms may play a role in developing insulin resistance. In the current study, we measured alternative splicing of insulin receptor in skeletal muscle from two dietary groups. Relative expression of insulin receptor mRNA isoforms in skeletal muscle was not affected by the composition of dietary fatty acids. Recently, we reported that the relative expression of insulin receptor mRNA isoforms was not altered in skeletal muscle of high-fat–fed, endurance-trained, and genetically NIDDM OLETF rats.[24,44,45] The present study suggests that mild hyperinsulinemia caused by a high-fat diet containing saturated fatty acids within a physiological range does not downregulate expression or alternative splicing of insulin receptor in skeletal muscle.

IRS-1 is the principal substrate phosphorylated by the insulin receptor tyrosine kinase. Tyrosine-phosphorylated IRS-1 then couples the insulin receptor to downstream signaling pathways by serving as a docking protein for signal molecules with *src* homology 2 domains.[16,17] PI 3-kinase, a heterodimer consisting of an 85-kd regulatory adaptor subunit tightly associated with a 110-kd catalytic subunit, is one of the insulin-signaling components that is activated by binding IRS-1 and has been implicated as part of the insulin signaling that triggers glucose transporter translocation, thus stimulating glucose transport.[46] Insulin-stimulated glucose uptake is significantly decreased in skeletal muscle from the obese and in adipose tissue from rats fed a high-fat diet, with reduced protein levels of IRS-1 and PI 3-kinase.[47,48] A recent study showed a reduction in IRS-1 protein in adipose tissue of insulin-resistant diabetic KKA^y mice[49] and in IRS-1 and PI 3-kinase protein in skeletal muscle of rats treated with dexamethasone.[50] In addition, mice made IRS-1–deficient by targeted gene knockout exhibit hyperinsulinemia and glucose intolerance.[51,52] These findings suggest that the decreased expression of IRS-1 and PI 3-kinase is involved in the developing insulin resistance. Therefore, we have investigated whether there is any alteration in the level of these putative mediators of the insulin-signaling cascade after long-term feeding of rats with safflower oil diet and a beef tallow diet. mRNA and protein levels of IRS-1 and PI 3-kinase were significantly lower in rats fed a beef tallow diet than in those fed a safflower oil diet. Our data suggest than an impairment of the initial steps in insulin signal-transduction pathways contributes to the defect in insulin-stimulated glucose uptake in skeletal muscle, thus resulting in insulin resistance.

There is increasing evidence that the impaired insulin signal transduction in hyperinsulinemic models of insulin

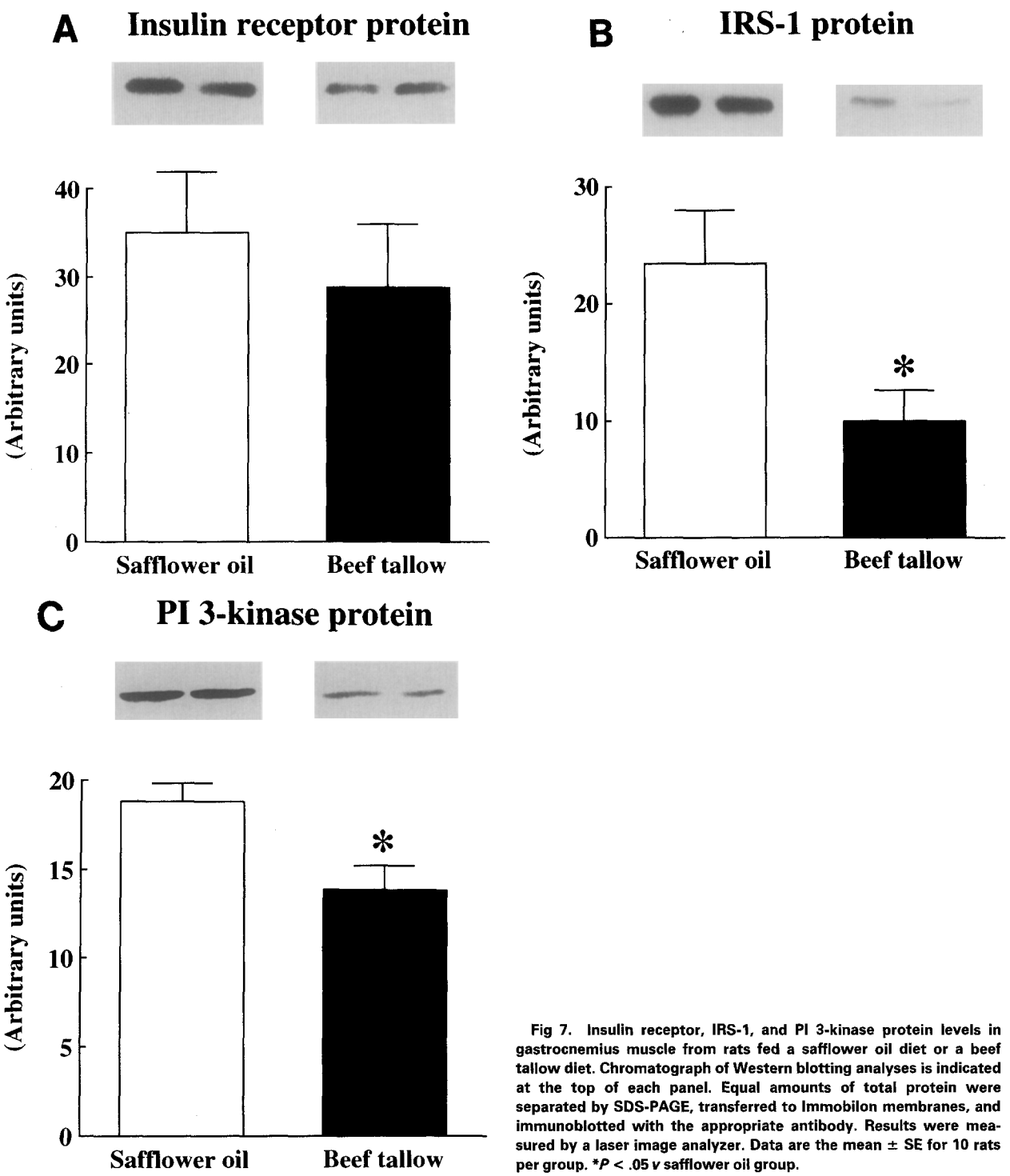

Fig 7. Insulin receptor, IRS-1, and PI 3-kinase protein levels in gastrocnemius muscle from rats fed a safflower oil diet or a beef tallow diet. Chromatograph of Western blotting analyses is indicated at the top of each panel. Equal amounts of total protein were separated by SDS-PAGE, transferred to Immobilon membranes, and immunoblotted with the appropriate antibody. Results were measured by a laser image analyzer. Data are the mean ± SE for 10 rats per group. *$P < .05$ *v* safflower oil group.

resistance extends to IRS-1 tyrosine phosphorylation and PI 3-kinase activation. A reduced tyrosine phosphorylation of IRS-1 in response to insulin has been described in adipocytes from patients with NIDDM,[53] skeletal muscle from obese human subjects[47] and the ob/ob mice,[27,54] and liver from dexamethasone-treated rats.[50] The blunted stimulation of PI 3-kinase by insulin is observed in skeletal muscle of gold thioglucose–obese mice[55,56] and obese subjects.[47] These results suggest that a reduction in the initial steps of insulin action after binding, IRS-1 phosphorylation, and activation of PI 3-kinase in target tissues may have an important role in the insulin resistance observed in these animals. Although we did not examine IRS-1 phosphorylation and PI 3-kinase activity in this study, it is possible that the reduced expression of IRS-1 and PI 3-kinase leads to a diminished IRS-1 tyrosine phosphorylation and PI 3-kinase activity in skeletal muscle of rats fed a beef tallow diet. Further studies are required to investigate how these early

insulin postreceptor events are regulated by insulin stimulation.

The decreased abundance of insulin receptor, IRS-1, and PI 3-kinase in the obese state is likely due to decreased transcription, since we recently found that levels of insulin receptor, IRS-1, PI 3-kinase, and MAP kinase mRNA in skeletal muscle were significantly lower in OLETF rats[45] (Nakajma R, Kim Y, Inoue T, et al, unpublished observation, January 1996). Furthermore, enhanced insulin sensitivity in skeletal muscle of endurance-trained rats is associated with increased levels of insulin receptor, IRS-1, PI 3-kinase, and MAP kinase mRNA[44] (Kim Y, Inoue T, NaKajma R, et al, unpublished observation, January 1996). Thus, early steps in the insulin signaling network play important roles in insulin resistance and other pathophysiologic states.

In conclusion, the results of this study indicate that long-term feeding of a high-fat diet with saturated fatty acids induces a decrease in IRS-1 and PI 3-kinase mRNA and protein levels, causing insulin resistance in skeletal muscle.

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