Polyunsaturated Fatty Acid-Mediated Suppression of Insulin-Dependent Gene Expression of Lipogenic Enzymes in Rat Liver

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Summary The effects of dietary polyunsaturated fat on insulin-dependent gene expression of lipogenic enzymes and a possible mechanism for PUFA-mediated suppression of the gene expression have been investigated in rat livers. When diabetic rats were injected with insulin, the insulin dose-dependent induction of lipogenic enzyme mRNAs were markedly reduced with increasing dietary corn oil. On the other hand, the PUFA-mediated suppression of the mRNA concentrations was partially restored by treatment with pioglitazone, a candidate for increasing insulin receptor phosphorylation. Moreover, insulin binding to receptors of liver, receptor autophosphorylation, and kinase activity toward exogenous substrate were lower in the corn oil diet group than in the hydrogenated fat group. The PUFA-mediated suppression of insulin binding was somewhat restored by pioglitazone, and the suppression of insulin receptor phosphorylation was significantly restored. It is suggested that the PUFA-mediated suppression of insulin-dependent gene expression of lipogenic enzymes can be ascribed to a decrease in insulin receptor binding primarily and also to receptor phosphorylation. Thus, PUFA appears to suppress the lipogenic enzyme gene expression stimulated by insulin.

Key Words lipogenic enzyme gene expression, pioglitazone, PUFAs, insulin receptors

The gene expression of lipogenic enzymes in the rat liver was increased by a fat-free/high-carbohydrate diet and decreased by feeding polyunsaturated fat, fasting or a diabetic state (1–5). The mRNA induction of all the lipogenic enzymes was remarkably reduced by PUFAs, but not by saturated or monounsaturated fatty

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Abbreviations: PUFA, polyunsaturated fatty acid; PMSF, phenylmethylsulfonyl fluoride; WGA, wheat germ agglutinin; Glu: Tyr, glutamine and tyrosine polymer.

Enzymes: acetyl-CoA carboxylase (EC 6.4.1.2), fatty acid synthase (EC 2.3.1.85), malic enzyme (EC 1.1.1.40), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), ATP-citrate lyase (EC 4.1.3.8).
acids (6, 7). Although the mechanisms of stimulation and suppression of lipogenic enzyme gene expression have scarcely been elucidated, the concept that the cis-acting elements of genes can be involved in the expression is generally accepted (8). Recently, it has been reported that the cis-linked PUFA response elements are localized in the promoter region of the L-pyruvate kinase gene and also in that of S14, which has been used as a model for examining the effects of PUFAs on hepatic lipogenic enzyme gene expression (9, 10). Moreover, a carbohydrate response element has already been found in the promoters of the L-pyruvate kinase and S14 genes (11–13). A similar nucleotide sequence also exists in the first intron of the fatty acid synthase gene (13). Recently, an insulin response element has been found in a rat fatty acid synthase promoter (14). However, the PUFA response elements have not been identified in any lipogenic enzyme gene.

On the other hand, we have recently found that lipogenic enzyme gene expression of genetically obese rats, which were less sensitive to insulin (15, 16), was not suppressed by PUFA as much as that of lean rats, possibly due to a defect of insulin binding to receptors (17). Cremel et al. (18) reported that linoleic acid decreased insulin binding to receptors in rat hepatoma cells, and tyrosine kinase activity of partially purified receptors from the cells decreased proportionally with the changes in insulin action. Therefore, the insulin receptor activity may be also involved in the PUFA-mediated suppression of lipogenic enzyme gene expression. To elucidate the mechanism, in the present experiment, we have investigated the effects of polyunsaturated fat feeding on the insulin-dependent gene expression in diabetic rat liver and the insulin receptor activity. Moreover, the effects of pioglitazone, a promising candidate for reducing insulin resistance (15, 16, 19–21), on the lipogenic enzyme gene expression and the PUFA-mediated suppression have been investigated. Subsequently, a possible mechanism for the PUFA-mediated suppression has been suggested.

MATERIALS AND METHODS

**Chemicals.** [α-32P]dCTP (111 TBq/mmol) and [γ-32P]ATP (167 TBq/mmol) were purchased from ICN. Nylon filters were purchased from Amersham (Hybond N). Other reagents were mostly from Sigma and Wako Pure Chemical Industries. Pioglitazone was a gift from Takeda Chemical Industries (Japan).

**Animals.** Male Wistar rats (SLC, Japan), 6 weeks old, were made diabetic by intravenous injection of streptozotocin (6 mg/100 g) after starvation for 20 h. Blood glucose was assayed 3 days after the treatment and rats with blood glucose levels of over 300 mg/dl were used for experiments. The animals were given a 5 or 10% fat diet (as indicated in the figure and table legends) for 5 days after the streptozotocin treatment. A fat-free diet contained 67% glucose, 18% casein, 9.9% cellulose, 5% salts, 0.1% choline chloride and vitamins (22). A part of the glucose was substituted by fat in the diet containing 5 or 10% fat. The animals were allowed to take water and diet ad libitum, but consumed similar amounts of the
diets among the dietary groups. The diabetic rats nibbled during day and night. They were kept under an automatic lighting schedule from 0800 to 2000 h at 24°C.

For insulin treatment, animals were subcutaneously injected with 0.625, 1.25, 2.50, or 3.75 U Lente insulin per 100 g body weight at 1700 h. The animals were killed 16 h after the insulin treatment. Pioglitazone, 5-[4-[2-(5-ethyl-12-pyridyl)-ethoxy]benzyl]-2,4-thiazolidinedione (3 mg/kg/day) was administered by orogastric tube for 5–7 days. A specimen of each liver was immediately frozen to measure the mRNA concentrations. Another specimen of the liver was used for the insulin receptor studies.

**Dot blot hybridization assay.** The cDNA species of lipogenic enzymes were cloned as described in our previous reports (1–5). Total RNA was isolated from the livers by the guanidium thiocyanate-phenol-chloroform extraction method (23). To measure the mRNA concentrations of lipogenic enzymes, the total RNA (5–10 µg) was denatured with formamide, spotted on nylon filters, and then irradiated with ultraviolet light for 5 min. The filter was prehybridized and then hybridized with 32P-labeled cDNAs as described previously (3). Relative densities of the hybridization signals were determined by scanning the autoradiograms at 525 nm (Model CS-9000, Shimadzu).

**Preparation of partially purified insulin receptors.** Insulin receptors were purified from livers essentially according to the method of Kadowaki et al. (24). The livers were homogenized in 3 volumes of 50 mM HEPES buffer (pH 7.6) containing 0.25 M sucrose, 1 µg/ml aprotinin, and 1 mM PMSF. The homogenate was centrifuged at 10,000 × g for 20 min, followed by centrifugation of the supernatant at 100,000 × g for 90 min. The pellet was suspended in 50 mM HEPES washing buffer (pH 7.6) containing 1 µg/ml aprotinin and 1 mM PMSF, and was washed twice at 100,000 × g for 60 min. The pellet was stored at −70°C for further purification. For experiments, samples were thawed and solubilized for 60 min in the presence of 2% Triton X-100. The insulin receptors were purified using a wheat germ lectin-agarose column. The procedures were carried out at 4°C.

**Insulin binding to receptors.** Lectin-purified insulin receptors were incubated with 125I-labeled insulin at 4°C for 16 h in the presence of various concentrations of unlabeled insulin in 200 µl of 25 mM HEPES (pH 7.6) containing 0.05% Triton X-100, 20 mM NaCl, 0.05 mg/ml bovine serum albumin, and 150 mM N-acetyl-D-glucosamine, essentially according to ref. (25). With human γ-globulin as carrier protein, receptor-bound insulin was precipitated with polyethylene glycol. Non-specific binding was defined as the radioactivity precipitated in the presence of 3.1 µM unlabeled insulin.

**Insulin receptor autophosphorylation and kinase activity.** Insulin receptors were purified from livers using wheat germ lectin-agarose as described by Freidenberg et al. (26). Autophosphorylation of the partially purified insulin receptors was performed by the method described in ref. (26). The receptors, containing 7 µg of protein, were preincubated with or without insulin at 4°C for 16 h in 40 µl of 25 mM HEPES buffer (pH 7.6) including 0.05% Triton X-100, 15 mM NaCl, 0.1 mg/ml
bovine serum albumin, and then incubated with 30 um [γ-32P]ATP, 2 mM MgCl₂, and 15 mM MnCl₂. After incubation at 4°C for 60 min, the reaction was terminated by the addition of fivefold-concentrated Laemmli sample buffer supplemented with ATP and dithiothreitol (27), and heating to 95°C for 3 min. Phosphorylated proteins were analyzed by 7.5% SDS-PAGE. The gels were stained, dried, and autoradiographed. Radioactivities incorporated into the 95,000-Mr protein in the gels were measured by cutting the appropriate regions from the dried gel slabs and counting with a liquid scintillation counter.

In the experiment measuring the insulin receptor kinase activity, after incubation of insulin receptors with varied concentrations of insulin, phosphorylation was initiated by adding [γ-32P] ATP, Mg²⁺, and Glu₄: Tyr₁. The reaction was terminated by the addition of unlabeled ATP (final concentration, 50 mM). Aliquots of the reaction mixtures were spotted on squares of Whatmann 3MM paper and washed in 10% trichloroacetic acid (28). The radioactivities of the dried papers were measured by scintillation counter.

Statistical analysis. Statistical evaluation of the results was carried out by Student’s t-test or by one-way analysis of variance (29).

RESULTS

Effects of dietary PUFA on insulin-dependent gene expression of lipogenic enzymes in rat liver

The mRNA concentrations of lipogenic enzyme set: acetyl-CoA carboxylase, fatty acid synthase, ATP-citrate lyase, malic enzyme, and glucose-6-phosphate dehydrogenase were very low in the livers of diabetic rats. However, the mRNA concentrations were restored to normal levels 16 h after injection of 2.5 U Lente insulin per 100 g body weight (1-4). To analyze the relative effects of insulin and PUFA on lipogenic enzyme gene expression, the mRNA concentrations in diabetic rats fed a 10% hydrogenated fat, 5% corn oil (plus 5% hydrogenated fat), or 10% corn oil diet were measured 16 h after injection of different doses of insulin (0.625-3.75 U/100 g). The mRNA concentrations in rats fed the 10% hydrogenated fat diet were increased by the insulin treatment to levels nearly equal to those in rats fed a fat-free diet (data not shown). However, the insulin-dependent increases of the mRNA concentrations in the 5% corn oil diet group were about half of those in the hydrogenated fat group, and were lower still in the 10% corn oil group. The results are shown in Fig. 1.

Comparison of the concentrations of acetyl-CoA carboxylase and fatty acid synthase mRNA after treatment with 2.5 U of insulin (per 100 g) in diabetic rats among the groups fed varied fat diets are shown in Table 1. The mRNA concentrations were not significantly decreased by hydrogenated fat or olive oil in comparison to the fat-free diet group, but were markedly decreased by fish oil or corn oil.
Fig. 1. Effects of insulin-treatment on mRNA concentrations of lipogenic enzymes in livers of diabetic rats fed corn oil. Streptozotocin diabetic rats fed on a 10% hydrogenated fat (triangle), 5% hydrogenated fat plus 5% corn oil (solid circle) or 10% corn oil (open circle) diet were injected with 0.625, 1.25, 2.50, or 3.75 U of Lente insulin per 100 g of body weight at 1700 h. The animals were killed at 900–1000 h the next morning. The mRNA concentrations are normalized against diabetic rats of the fat-free diet group. A: Acetyl-CoA carboxylase, B: fatty acid synthase, C: ATP-citrate lyase, D: malic enzyme, E: glucose-6-phosphate dehydrogenase. Significantly different among the dietary groups at 1.25, 2.50, and 3.75 U insulin treatment, at p < 0.05 at least. Mean ± SD (n = 12).

Table 1. Effects of dietary fat type and pioglitazone treatment on lipogenic enzyme mRNA concentrations in the rat liver.

| Diet                      | Acetyl-CoA carboxylase (fold) | Fatty acid synthase (fold) |
|---------------------------|-------------------------------|----------------------------|
| Fat-free                  | 1.00 ± 0.23<sup>a</sup>       | 1.00 ± 0.12<sup>a</sup>    |
| Hydrogenated fat          | 0.88 ± 0.04<sup>a</sup>       | 0.81 ± 0.11<sup>a</sup>    |
| Hydrogenated fat + pioglitazone<sup>1</sup> | 0.76 ± 0.07<sup>a</sup>       | 0.77 ± 0.11<sup>a</sup>    |
| Olive oil                 | 1.05 ± 0.25<sup>a</sup>       | 0.78 ± 0.16<sup>a</sup>    |
| Corn oil                  | 0.55 ± 0.02<sup>b</sup>       | 0.29 ± 0.04<sup>b</sup>    |
| Corn oil + pioglitazone   | 0.77 ± 0.16<sup>**</sup>      | 0.41 ± 0.07<sup>**</sup>   |
| Fish oil                  | 0.35 ± 0.12<sup>b</sup>       | 0.21 ± 0.01<sup>b</sup>    |

Streptozotocin diabetic rats fed on a fat-free or various kinds of fat diet for 5 days were injected with Lente insulin (2.5 U/100 g body weight) at 1700 h and killed at 900–1000 h the next morning. Pioglitazone (3 mg/kg) was administered by orogastric tube each day for 5–7 days. The mRNA concentrations were normalized to the fat-free diet. Means with different superscript letters are significantly different among each of the dietary groups (p < 0.05). *Significantly different from no pioglitazone treatment (p< 0.05). Mean ± SD (n = 5–6).

Effects of pioglitazone on mRNA concentrations

After diabetic rats fed corn oil were treated with insulin, the PUFA-mediated suppression of mRNA concentrations of acetyl-CoA carboxylase and fatty acid
Effects of pioglitazone on insulin binding to receptors and receptor phosphorylation

The insulin binding to receptors was lower in the corn oil diet group than in the hydrogenated fat group (Fig. 2). Pioglitazone treatment did not increase insulin binding in the hydrogenated fat group but did in the corn oil group. However, the Scatchard plots revealed that insulin binding affinity to receptors was not affected by dietary fat type or by pioglitazone treatment.

The insulin-stimulated autophosphorylation of the β-subunit of insulin receptors of livers was significantly decreased by corn oil feeding in comparison with the hydrogenated fat diet group, as shown in Fig. 3 (upper). The PUFA-mediated suppression was partially restored by the pioglitazone treatment. Moreover, a similar result was observed in the insulin receptor kinase activities with exogenous substrate (Glu4:Tyr1). The results of 32P incorporation to the substrate are shown in Fig. 3 (lower).

DISCUSSION

When diabetic rats adapted to a 10% hydrogenated fat, 5% corn oil (+5% hydrogenated fat) or 10% corn oil diet were injected with insulin, the insulin...
Autophosphorylation of insulin receptors (A) and phosphorylation of Glu4: Tyr by insulin receptors (B) of rat livers. (A) The lectin-purified insulin receptors of diabetic rat livers were preincubated with different concentrations of insulin and then incubated with $[\gamma^{32}P]ATP$. The samples were subjected to SDS-PAGE. $^{32}P$ incorporation into the $\beta$-subunit of insulin receptors (95,000-$M_\text{r}$) was measured for autophosphorylation. (B) After incubation of insulin receptors with different concentrations of insulin, phosphorylation was initiated by adding $[\gamma^{32}P]ATP$, Mg$^{2+}$, and Glu4: Tyr1. The circles show data for the hydrogenated fat diet group and the triangles for the corn oil group. Solid symbols of each group show data for pioglitazone treatment. Means with different superscript letters are significantly different among data at 1, 10, or 100 nM insulin treatment ($p<0.05$). Experiments were performed using 6 animals for each group (Mean±SD).

dose-dependent inductions of lipogenic enzyme mRNAs were markedly reduced with increasing dietary corn oil in a dose-dependent fashion. The PUFA-mediated suppression of the mRNA concentrations was partially restored by pioglitazone treatment. Sugiyama et al. (15) and Ikeda et al. (16) reported that the effects of
pioglitazone on insulin-dependent glucose oxidation and lipogenesis seem to be due to increased insulin sensitivity. However, the effects were not accompanied by any changes in insulin binding, suggesting that pioglitazone can improve glucose and lipid metabolism by reducing insulin resistance on the post-binding system (15). Kobayashi et al. (19) also reported that pioglitazone treatment did not change insulin binding to receptors but increased the insulin receptor autophosphorylation and kinase activity toward exogenous substrate, suggesting that pioglitazone increase insulin sensitivity in part by activating kinase of the receptors. Cremel et al. (18) reported that linoleic acid decreased the insulin binding to receptors in rat hepatoma cells, and tyrosine kinase activity of partially purified receptors from the cells was also decreased with the changes in insulin action.

On the other hand, we have recently found that suppression of lipogenic enzyme gene expression by PUFA in genetically obese rats, which were less sensitive to insulin (15,16), was less than that of lean rats, possibly due to a defect of insulin binding to receptors (17). It was suggested that PUFA-mediated regulation of lipogenic enzyme gene expression may be involved in the function of insulin receptors.

In the present experiment, we have found that the insulin binding to receptors and also insulin receptor autophosphorylation and kinase activities toward exogenous substrate were decreased by feeding polyunsaturated fat. The PUFA-mediated suppression of insulin receptor autophosphorylation and kinase activities was partially restored by pioglitazone treatment. The PUFA-mediated suppression of insulin binding to receptors was also restored by pioglitazone treatment. The pioglitazone treatment also partially restored the PUFA-mediated suppression of lipogenic enzyme gene expression. As a result of the present study and previous studies (16–19), it is suggested that PUFA-mediated suppression of insulin-dependent gene expression of lipogenic enzymes can be primarily ascribed to insulin binding to receptors, and also to insulin receptor phosphorylation and/or signal transduction. Thus, it is postulated that the PUFA-mediated suppression of the insulin-dependent lipogenic enzyme gene expression can by involved in the function of insulin receptors. Moreover, PUFA appears to suppress the lipogenic enzyme gene expression stimulated by insulin.

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