Alterations in insulin-induced postreceptor signaling in adipocytes of the Otsuka Long-Evans Tokushima fatty rat strain

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Abstract

The Otsuka Long-Evans Tokushima fatty (OLETF) rat is a new spontaneous non-insulin-dependent diabetes mellitus (NIDDM) model rat strain developed in Tokushima, Japan. After 18 weeks of age, decreases of 45% and 40% respectively in insulin- and phorbol ester-stimulated [3 H]2-deoxyglucose (DOG) uptake were observed, compared with those in Long-Evans Tokushima (LETO) rats (control). Insulin-specific binding and 95 kDa autophosphorylation of insulin receptor in OLETF rats were not different from those in LETO rats. Insulin-induced diacylglycerol (DG) production and Mono Q column-purified protein kinase C (PKC) translocation in adipocytes of OLETF rats were decreased compared with those of LETO rats. Insulin-induced PKC β translocation from cytosol to membrane was also decreased in adipocytes of

OLETF rats. Increases of the PKC β I, β II, ϵ and ζ isoforms in membranes of OLETF rats were markedly smaller than those of LETO rats. Analysis of mRNA levels of PKC isoforms in adipocytes of OLETF rats showed decreases of basal level and insulin-induced delayed responses of PKC β I, β II, ϵ and ζ mRNA in OLETF rats. On the other hand, insulin- or phorbol ester-induced phosphatidylinositol 3-kinase (PI 3-kinase) activation was decreased in adipocytes of OLETF rats compared with those of LETO rats. These results suggest that insulin resistance in OLETF rats, a spontaneous NIDDM model rat, may be associated with deterioration of insulin-induced DG-PKC signaling and subsequent decrease in PI 3-kinase activation.

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Introduction

Activation of protein kinase C (PKC) is a major mechanism for transduction of signals (Nishizuka 1988). Agonists such as hormones, neurotransmitters, and growth factors stimulate the hydrolysis of various phospholipids in the plasma membrane to generate diacylglycerol (DG) which causes PKC to translocate from the cytosol to specific membrane sites. In particular, insulin and phorbol esters provoke increases in membrane-associated PKC in rat adipocytes (Ishizuka *et al.* 1989, Egan *et al.* 1990). Moreover, it has previously been reported that PKC β is translocated from cytosol to membrane in response to treatment of rat adipocytes (Ishizuka *et al.* 1989) and soleus muscle (Ishizuka *et al.* 1990, 1991) with insulin and/or 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). However, it is known that cells contain various PKC isoforms (Ishizuka *et al.* 1992).

It is also well accepted that phosphatidylinositol 3-kinase (PI 3-kinase) is associated with and activated by a number of proteins containing tyrosine kinase activities, including the receptor for platelet-derived growth factor (Whitman *et al.* 1987), insulin (Endeman *et al.* 1990,

Ruderman *et al.* 1990), and the products of oncogenes (Varticovski *et al.* 1989). PI 3-kinase, which consists of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (Carpenter & Cantley 1990, Escobedo *et al.* 1991, Hiles *et al.* 1992), is believed to play important roles in the transduction of mitogenic signals (Cantley *et al.* 1991). Although receptor-mediated activation of PI 3-kinase was also found in N-formyl-Met-Leu-Phe (fMLP)-stimulated human neutrophils (Traynor-Kaplan *et al.* 1989) and thrombin-stimulated human platelets (Kucera & Rittenhouse 1990), the physiological role of PI 3-kinase in these cells is still uncertain.

Despite the physiological importance of insulin sensitive tissues such as liver, muscle and adipose tissue, much remains unknown about the post-receptor signaling system in these tissues. Elucidation of the signal pathway is especially relevant in understanding the mechanism underlying insulin resistance in skeletal muscle and adipocytes. One such pathological state is the obese, hyperinsulinemic syndrome that is commonly associated with non-insulin-dependent diabetes mellitus (NIDDM). Previously, we reported that insulin-induced PKC activation in adipocytes of old-obese rats was decreased,

Table 1 Primers used in mRNA amplification

Oligonucleotide	Sequences	Fragment size (bases)
PKCβI-5'	CTTTGGCATGTGTAAAGAGAATATCTGGGA	442
PKCβI-3'	GTCGAAGTTGGAGGTGTCTCGCTTGTCTCT	
PKCβII-5'	CTTTGGCATGTGTAAAGAGAATATCTGGGA	571
PKCβII-3'	TTAGCTCTTGACTTCGGGTTTTAAAAATTC	
PKCε-5'	TCATCGATCTCTCGGGATCATCGG	733
PKCε-3'	CGGTTGTCAAATGACAAGGCATTC	
PKCζ-5'	CGATGGGATGGATGGGATCAAAATC	685
PKCζ-3'	TTCAGTATTCATGTCAGGGTTGTCT	
Hprt-5'	GTTGGATACAGGCCAGACTTTGTTC	162
Hprt-3'	GATTCAACTTGGCGCTCATCTTAGGC	

Table 2 General characteristics of OLETF and LETO rats

Rat strain	Age (week)	Body weight (g)	Plasma glucose (mM)	IRI (pM)
OLETF	8	270 ± 38	7.0 ± 2.6	120 ± 23
	12	395 ± 42	7.5 ± 2.4	144 ± 26
	18	510 ± 60*	11.2 ± 3.6*	250 ± 38*
	24	590 ± 68**	12.3 ± 4.1**	403 ± 62**
	30	625 ± 79**	14.3 ± 5.1**	450 ± 71**
LETO	8	225 ± 37	6.7 ± 2.3	115 ± 28
	12	348 ± 46	6.8 ± 2.6	122 ± 32
	18	422 ± 48	6.9 ± 2.8	183 ± 40
	24	485 ± 53	7.0 ± 3.1	250 ± 42
	30	496 ± 56	7.1 ± 2.9	270 ± 63

*P<0.05, **P<0.01 compared with LETO (Student's t-test).

compared with that in young-lean rats (Ishizuka *et al.* 1993). Moreover, a defect in skeletal muscle PI 3-kinase in obese insulin-resistant mice was also reported (Heydrick *et al.* 1993).

A spontaneous long-term hyperglycemic rat with diabetic complications, the Otsuka Long-Evans Tokushima fatty (OLETF) strain has been established (Kawano *et al.* 1992). Clinical and pathological features of disease in OLETF rats resemble those of human NIDDM. However, in the OLETF rat abnormalities of insulin-induced postreceptor signaling remain unclear. In the present study, we have shown that the insulin-induced DG-PKC signaling was diminished in adipocytes of OLETF rats.

Materials and Methods

Materials

Pork insulin was obtained from Novo (Copenhagen, Denmark). [γ -³²P]ATP (3000 Ci/mmol) and [1,2-³H]2-deoxyglucose ([³H]2-DOG), [2-³H]glycerol (5 Ci/mmol), [9,10-³H]palmitic acid (30–60 Ci/mmol) and L-[1-¹⁴C]glucose (47 mCi/mmol) were purchased from New

England Nuclear (Boston, MA, USA). Phosphatidylserine (PS), diolein, histone (type III-S), phenylmethylsulfonyl fluoride (PMSF), leupeptin, 12-O-tetradecanoyl phorbol-13-acetate (TPA), BSA, D-glucose, ATP and goat anti-rabbit γ -globulin complexed to alkaline phosphatase were purchased from Sigma Chemical Co. (St Louis, MO, USA). Silicon oil was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). PKC β , ϵ and ζ antibodies were purchased from Gibco BRL Products (Grand Island, NY, USA). PKC β I and β II were purchased from Santa Cruz Co (CA, USA). All other chemicals were of reagent grade or better.

Adipocyte experiments

Male OLETF rats, an animal model of human NIDDM, and Long-Evans Tokushima (LETO) rats, achieved by selective breeding of the control line which was obtained by different original matings from those for OLETF rats, both strains originating from the same colony of Long-Evans rats (Kawano *et al.* 1992), were allowed to feed *ad libitum* and were killed by decapitation. Free adipocytes were isolated by collagenase digestion of rat epididymal fat

Figure 1 Insulin- or TPA-stimulated [3 H]2-DOG uptake in adipocytes of OLETF and LETO rats at 18 weeks of age. Isolated adipocytes were washed and preincubated in glucose-free KRP containing 1% BSA for 30 min. [3 H]2-DOG was then added to a cell suspension, and uptake of [3 H]2-DOG was measured over 1 min. Unstimulated [3 H]2-DOG uptake is shown as Control. [3 H]2-DOG uptake in adipocytes of OLETF (open bars) and LETO rats (solid bars) at 18 weeks of age during stimulation with 10 nM insulin and 1 μ M TPA for 30 min was measured. Results are expressed as pmol glucose/mg protein and represent three to four separate experiments, each conducted in triplicate. Values are means $\pm$ S.E. * $P < 0.01$ (Student's *t*-test).

pads (Rodbell 1964) in Krebs-Ringer phosphate (KRP) buffer (pH 7.4) containing 127 mM NaCl, 12.3 mM NaH_2PO_4 , 5.1 mM KCl, 1.3 mM MgSO_4 , 1.4 mM CaCl_2 , 3% BSA, and 2.5 mM glucose. Adipocytes were washed and preincubated at 37 °C in glucose-free KRP buffer containing 1% BSA for 30 min, and then incubated with or without 10^{-6} M TPA, 10 nM insulin for 30 min. [3 H]2-DOG (0.08 μ Ci) and unlabeled 2-DOG (0.05 mM) were then added to 300 μ l of a 10% (vol/vol) adipocyte suspension, and uptake of [3 H]2-DOG was measured over 1 min (Olefsky 1978). In PKC experiments, reactions were terminated by addition of 10 ml ice-cold buffer I (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 0.1 mM PMSF, 20 μ g/ml leupeptin, 20 mM mercaptoethanol) at the indicated time. The adipocytes were washed twice and homogenized in buffer I. Homogenates were centrifuged at 1000 *g* for 2 min, and floating fatty materials were removed. Resultant homogenates were centrifuged at 105 000 *g* for 60 min to obtain cytosol and membrane fractions as described below.

Insulin binding studies

Isolated adipocytes were incubated with ^{125}I -insulin (2000 Ci/mmol, Amersham, Tokyo, Japan) and unlabeled insulin (1 to 1000 nmol/l) in plastic tubes at 25 °C in a shaking water bath for 60 min. Incubations were terminated by removing 300 μ l aliquots from the cell suspension and rapidly centrifuging the cells in plastic microfuge tubes

to which 100 μ l silicone oil had been added. The cells were then removed, and the radioactivity was determined. All studies were performed in triplicate.

Autophosphorylation of insulin receptor

Isolated adipocytes were homogenized in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mg/ml Bacitracine, 20 mM (p-amidinophenyl)-methanesulphonyl fluoride hydrochloride, 5 mM EDTA, 5 mM EGTA, 20 mM sodium pyrophosphate, 1 mM orthovanadate, 20 mM NaF), and then centrifuged at 15 000 r.p.m. for 20 min to obtain the supernatant. The resultant supernatant was placed on an anti-insulin receptor antibody-coated U-bottom plate (Ebina *et al.* 1987) and incubated for 4 h. After washing the insulin receptor four times, 20 μ l 10^{-6} M insulin and kinase buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl_2 , 5 mM MnCl_2 , 1 μ M [γ - ^{32}P]ATP) were added to the insulin receptor and incubated for 60 min at room temperature. The sample was subjected to SDS-PAGE and autoradiographed to obtain 95 kDa phosphorylating activity.

PKC studies

Rat adipocytes isolated as above were homogenized with a polytron homogenizer in buffer I. The homogenates were centrifuged for 60 min at 105 000 *g* to separate the cytosol and membrane fractions. After membranes were resuspended in buffer I containing 5 mM EGTA, 2 mM EDTA, and 1% Triton X-100 for 30 min at 4 °C, they were sonicated, and centrifuged at 105 000 *g* to obtain solubilized membrane fractions. To measure PKC enzyme activity of adipocytes, cytosol or solubilized membrane fractions were diluted with 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol (buffer II). The samples were then applied to a Mono Q column (0.5 $\times$ 0.5 cm, Pharmacia HR 5/5) that was equilibrated with buffer II and connected to a high performance liquid-chromatography system, as described previously (Ishizuka *et al.* 1989, 1990). PKC was eluted by application of a 20 ml linear gradient of NaCl (0–0.7 M) in buffer II at a flow rate of 0.65 ml/min. Fractions of 1 ml were collected, and PKC activity of each fraction was assayed by measuring the phosphorylation of histone III-S, as described previously (Ishizuka *et al.* 1989).

Activation of PKC in rat adipocytes was also assayed by changes in the subcellular distribution of immunoreactive PKC using the methods described previously (Ishizuka *et al.* 1989, 1990). Equal amounts of cytosol or membrane fractions were prepared as described above and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and incubated first with rabbit polyclonal antiserum raised to

Figure 2 Insulin-specific binding and insulin receptor tyrosine kinase activity in adipocytes of OLETF and LETO rats at 18 weeks of age. Isolated adipocytes (10% cell suspension) were incubated with ^{125}I -insulin in various concentrations of unlabeled insulin (1 to 1000 nM) at 25 °C for 60 min. Cells specifically binding ^{125}I -insulin were removed from the cell suspension as indicated in Materials and Methods, radioactivity was counted, and insulin specific binding was analyzed by Scatchard analysis (lower left panel). Autophosphorylation of insulin receptor β subunit was measured using immunoprecipitation with anti-insulin receptor antibody as described in Materials and Methods (right panel). Densitometric data are means $\pm$ S.E. of three separate determinations (upper left panel).

synthetic peptide specific to each PKC β , ϵ , ζ (Gibco BRL, Grand Island, NY, USA), βI , βII (Santa Cruz, CA, USA) and secondly with goat anti-rabbit γ -globulin complexed to alkaline phosphatase. Specificities for PKC β , βI , βII , ϵ , and ζ antibodies were verified by added immunogenic peptide. As reported (Ishizuka *et al.* 1989), this immunoblotting method detected a single major immunoreactive band that comigrated on SDS-PAGE and blotted identically with purified rat brain 80 000- M_r (PKC β , βI , βII and ζ) and 90 000- M_r (PKC ϵ) PKCs. The intensity of immunoreactivity was scanned with a laser densitometer (Pharmacia LKB Biotechnology, Tokyo, Japan) to determine the relative value.

Incorporation of [^3H]glycerol into diacylglycerol

Adipocytes were incubated for 30 min in 0.5 ml KRP buffer. [^3H]Glycerol (10 μCi) or [^3H]palmitic acid was then added, and after prelabeling adipocytes for 15 min or

60 min, vehicle (controls) or insulin was added, and the incubation was continued for the indicated times. Reactions were stopped by the addition of methanol (final concentration, 50%). Samples were transferred to glass tubes. Chloroform (2 vol) was added, and extraction of the lipids was performed as described previously (Hoffman *et al.* 1991).

Diacylglycerol content in adipocytes

Diacylglycerol content was measured by the method of Preiss *et al.* (1986). The reaction was stopped by the addition of methanol (final concentration, 50%). Samples were transferred to glass tubes, and cell suspensions were homogenized with a sonicator. Chloroform (2 vol) was added, and extraction of the lipids was performed. The resultant samples were washed, evaporated, sonicated in 20 μl solution I (7.5% octyl- β -glucoside, 5 nM cardiolipin and 1 mM) diethylene triaminepentaacetic acid

Table 3 Effect of insulin on [^3H]glycerol incorporation into DG in adipocytes of OLETF and LETO rats at 18 weeks of age. Adipocytes were prelabeled for 15 min or 60 min with 10 $\mu\text{Ci/sample}$ [^3H]glycerol (a) or 5 $\mu\text{Ci/sample}$ [^3H]palmitic acid (b), and then incubated in the presence or absence of insulin (10 nM) for the times indicated. Insulin-induced alterations of DG content (c) were also measured as indicated in Materials and Methods. Results are depicted as a percentage of time-matched control values (in parentheses) and represent three separate experiments, each conducted in triplicate (means $\pm$ S.E.)

	OLETF rats (c.p.m./mg protein)	LETO rats (c.p.m./mg protein)
Time (min)		
(a) Insulin-stimulated [^3H]glycerol incorporation into diacylglycerol		
Basal	56 951 $\pm$ 5436 (100%)	47 154 $\pm$ 6832 (100%)
2	66 062 $\pm$ 8544 (116%)	64 600 $\pm$ 7074 (137%)
5	80 302 $\pm$ 11 390 (141%)	61 302 $\pm$ 7544 (130%)
10	47 839 $\pm$ 6266 (84%)*	75 446 $\pm$ 8958 (160%)
20	61 508 $\pm$ 2279 (108%)*	95 249 $\pm$ 8488 (202%)
(b) Insulin-stimulated [^3H]palmitate incorporation into diacylglycerol		
Basal	67 862 $\pm$ 6413 (100%)	72 967 $\pm$ 6975 (100%)
2	83 695 $\pm$ 8144 (123%)	96 351 $\pm$ 10 214 (132%)
5	72 341 $\pm$ 6787 (106%)	77 344 $\pm$ 9484 (106%)
10	87 187 $\pm$ 11 334 (128%)	107 993 $\pm$ 10 944 (148%)
20	87 498 $\pm$ 9502 (129%)*	149 586 $\pm$ 8758 (205%)
(c) diacylglycerol content		
	OLETF rats (^{32}P c.p.m./mg protein)	LETO rats (^{32}P c.p.m./mg protein)
Time (min)		
Basal	10 787 $\pm$ 721 (100%)	10 947 $\pm$ 733 (100%)
2	12 498 $\pm$ 1295 (116%)*	21 347 $\pm$ 1533 (195%)
5	6793 $\pm$ 1403 (63%)	10 070 $\pm$ 1642 (92%)
10	12 698 $\pm$ 862 (118%)	16 421 $\pm$ 1203 (150%)
20	16 849 $\pm$ 1511 (156%)*	36 674 $\pm$ 2081 (335%)

* $P < 0.05$ – 0.01 compared with LETO rats (Student's *t*-test).

(DETAPAC), and then incubated for 30 min at 25 °C after the addition of 50 μl solution II (100 mM imidazol-HCl, pH 6.6, 100 mM NaCl, 25 mM MgCl_2 , 2 mM EGTA), 20 μl solution III (2.2 mM dithiothreitol, 0.1 mg/ml DG kinase; Calbiochem Co., San Diego, CA, USA) and 10 μl solution IV (1 mM [γ - ^{32}P]ATP, 100 mM imidazol and 1 mM DETAPAC). The reaction was stopped with 2 ml chloroform/methanol (2:1) and then washed twice with chloroform and 1 M NaCl. Aliquots were analyzed by TLC on silica gel plates, developed in chloroform/methanol/acetic acid (65:15:5) and products detected by autoradiography to obtain phosphatidic acid formation by DG kinase.

PKC mRNA experiment

Total RNA was prepared using ISOGEN (Nippon Gene Inc., Toyama, Japan). In general 1–2 ml of packed adipocytes were lysed in 0.5 ml ISOGEN solution,

Figure 3 Insulin-induced translocation of Mono Q column-purified PKC activity in adipocytes of OLETF and LETO rats at 18 weeks of age. Cytosolic and membrane-associated PKC activities were measured as indicated in Materials and Methods. PKC activity was expressed as the percentage of unstimulated PKC activity (control: C). Values are means $\pm$ S.E. of three separate experiments. * $P < 0.05$ vs LETO-INS 10 (Student's *t*-test).

Figure 4 Insulin- and TPA-induced redistribution of PKC β immunoreactivity in adipocytes of OLETF and LETO rats at 18 weeks of age. Cytosolic and membrane-associated proteins (30 μ g) were subjected to SDS-PAGE, transferred, and analyzed as indicated in Materials and Methods. (a) Representative experiment. (b) Densitometric data of PKC β immunoreactivity in adipocytes of OLETF (●, ■) and LETO (○, □) rats. Cytosolic protein (squares), membrane-associated protein (circles). Results are means $\pm$ S.E. of three experiments. ** $P < 0.01$, * $P < 0.05$ vs OLETF rats.

followed by chloroform extraction and isopropanol/ethanol precipitation. First strand cDNA synthesis was performed according to the method of Frohman *et al.* (1988). At the end of the incubation period, samples were diluted with 80 μ l Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Aliquots of 10 μ l of first strand cDNA were mixed with 40 μ l PCR mix (Takara Shuzo Co., Tokyo, Japan). Primers specific for the different PKC isozymes were designed based on reported sequences from the Gene Bank data base as indicated in Table 1. The location of the primers in the cDNA sequences was selected in such a way that they would target the specific region (Ono *et al.* 1989). Hypoxanthine phosphoribosyl-

transferase (Hprt) mRNA level was used as a control and was not affected by insulin or phorbol ester. PCR amplification was carried out using a Perkin-Elmer thermal cycle set for 30 to 40 cycles. The temperatures used for PCR were: denaturing 94 $^{\circ}$ C, 1 min; annealing 55 $^{\circ}$ C, 2 min; primer extension 72 $^{\circ}$ C, 3 min. PCR fragments were analyzed by electrophoresis on 1.5% agarose gel, and DNA was visualized by ethidium bromide staining.

PI 3-kinase assay

Isolated adipocytes were treated with or without insulin (10 nM) for 0, 1, 5, 10 and 60 min at 37 $^{\circ}$ C, lysed in

Figure 5 (a) Immunoblot analysis of insulin-induced translocation of PKC β , β II, ϵ and ζ in adipocytes of OLETF and LETO rats. Cytosolic and membrane-associated proteins (30 μ g) were subjected to SDS-PAGE, transferred, and analyzed as indicated in Materials and Methods. (b) Densitometric data of PKC isoforms in adipocytes of OLETF (●, ■) and LETO (○, □) rats. Cytosolic protein (squares), membrane-associated protein (circles). Results are means $\pm$ S.E. of three experiments. ** $P < 0.01$, * $P < 0.05$ vs OLETF rats.

(a) Immunoblot analysis of PKC β in rat adipocytes

Immunoblot analysis of PKC β II in rat adipocytes

Immunoblot analysis of PKC ϵ in rat adipocytes

Immunoblot analysis of PKC ζ in rat adipocytes

(b) Insulin-induced PKC β changes

Insulin-induced PKC β II changes

Insulin-induced PKC ϵ changes

Insulin-induced PKC ζ changes

buffer containing 1% (vol/vol) Nonidet P-40 (Hayashi *et al.* 1992), and immunoprecipitated with an anti-phosphotyrosine antibody (Kanai *et al.* 1993) and protein A-agarose. The immunoprecipitates were washed and subjected to the PI 3-kinase assay as described (Hayashi *et al.* 1992). Briefly, cells were treated with 10 nM insulin for the indicated periods and lysed in the buffer (20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5 mM EDTA, 5% glycerol, 1 mM orthovanadate, and 20 μ M p-amidinophenylmethanesulfonyl fluoride hydrochloride) and sonicated. The homogenates were centrifuged at 15 000 r.p.m. for 20 min, and the indicated supernatants were incubated with 4 μ g anti-phosphotyrosine antibody for 2 h at 4 °C. The immunocomplexes were precipitated with 40 μ l protein A-agarose and washed twice. The immunoprecipitates were subjected to the PI kinase assay in a 50- μ l reaction mixture containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM EGTA, 100 μ M PI, 100 μ M phosphatidylserine, and 10 μ M [γ -³²P]ATP (0.1 μ Ci/ μ l). After 10 min at 30 °C the reaction was stopped by adding 200 μ l 1 M HCl and 80 μ l chloroform/methanol (1:1, vol/vol). A 30 μ l portion of the lower layer was spotted onto a silica gel 60 plate (Merck) and developed in chloroform/methanol/25% NH₄Cl/water (43:38:5:7, vol/vol). The radioactive PI phosphate spot was detected by autoradiography, scraped from the plate and quantified by liquid scintillation counting.

Immunoblot

Immunoprecipitates with the anti-phosphotyrosine antibody were separated by SDS-PAGE and transferred onto nitrocellulose paper. The paper was blocked with 3% gelatin TBS and incubated with the anti-p85 PI 3-kinase antibody (1:1000 dilution) for 4–5 days. Protein bands were located using the Enhanced ChemiLuminescence (ECL) system.

Statistical comparisons were performed by standard Student's *t*-test for planned paired comparisons where appropriate. Unless otherwise stated, all data are expressed as means $\pm$ S.E.

Results

Clinical profiles of OLETF and LETO rats from 8 to 30 weeks of age

Body weight, fed plasma glucose and immunoreactive insulin (IRI) levels are shown in Table 2. Up to 12 weeks,

there was no significant difference in any of these values, whereas after 18 weeks significant increases in plasma glucose and IRI levels were observed in OLETF rats compared with LETO rats.

Insulin- or phorbol ester-stimulated glucose uptake in adipocytes of OLETF and LETO rats

Insulin (10 nM)-stimulated [³H]2-DOG uptake in adipocytes of OLETF rats at 12 weeks of age was slightly lower compared with that in adipocytes of LETO rats. A phorbol ester, TPA-stimulated [³H]2-DOG uptake in adipocytes of OLETF rats at 12 weeks of age was also slightly decreased compared with that in adipocytes of LETO rats (data not shown).

At 18 weeks of age, 10 nM insulin- or 1 μ M TPA-stimulated [³H]2-DOG uptake in adipocytes of OLETF rats was apparently decreased compared with that of LETO rats (insulin-stimulated [³H]2-DOG uptake: OLETF 72.7 $\pm$ 6.6 pmol/mg protein vs LETO 164.3 $\pm$ 8.8 pmol/mg protein, *P* < 0.01; TPA-stimulated [³H]2-DOG uptake: OLETF 40.5 $\pm$ 4.9 pmol/mg protein vs LETO 82.5 $\pm$ 5.8 pmol/mg protein, *P* < 0.01; unstimulated [³H]2-DOG uptake: OLETF 31.7 $\pm$ 4.4 pmol/mg protein, LETO 38.6 $\pm$ 7.6 pmol/mg protein) as indicated in Fig. 1.

Insulin-specific binding to insulin receptor and autophosphorylation of insulin receptor in adipocytes of OLETF and LETO rats

After 18 weeks of age, ¹²⁵I-insulin binding activity and 95 kDa tyrosine kinase activity of the insulin receptor β subunit in adipocytes of OLETF rats was not significantly different from those of LETO rats (Fig. 2). Densitometric analysis of 95 kDa autophosphorylation (right panel in Fig. 2) in adipocytes of OLETF rats was not changed compared with those of LETO rats (upper left panel in Fig. 2).

Insulin-induced [³H]diacylglycerol production and diacylglycerol content in adipocytes of OLETF and LETO rats

After stimulation with 10 nM insulin, [³H]DG production was increased by 5 min, then decreased to the unstimulated level, and increased by 20 min in adipocytes of OLETF and LETO rats at 12 weeks of age (data not shown). On the other hand, [³H]glycerol incorporation into DG in adipocytes of OLETF rats at 18 weeks of age

Figure 6 Expression of (a) PKC β I, β II, ϵ , ζ isoforms and (b) Hprt in adipocytes of OLETF and LETO rats. Total RNA (0.5 μ g) from isolated adipocytes was reverse-transcribed and aliquots of cDNA pools were obtained after the reaction was terminated by the addition of 0.5 ml ISOGEN to insulin-stimulated packed adipocytes (1 ml) at each of the indicated times. Agarose gel electrophoresis of the PCR products generated after 35 cycles of amplification of rat adipocytes cDNA using the pairs of oligonucleotide primers was carried out as indicated in Materials and Methods. (c) Densitometric data of PKC isoform mRNA in adipocytes of OLETF (●) and LETO (○) rats. Results are means $\pm$ S.E. of three separate experiments. ***P* < 0.01, **P* < 0.05 vs OLETF rats.

(a) Insulin-induced PKC β I m-RNA change in rat adipocytes

Insulin-induced PKC β II m-RNA change in rat adipocytes

Insulin-induced PKC ϵ m-RNA changes in adipocytes of OLETF and LETO rats

Insulin-induced PKC ζ m-RNA change in rat adipocytes

(c) Insulin-induced PKC β I m-RNA change

Insulin-induced PKC β II m-RNA change

Insulin-induced PKC ϵ m-RNA change

Insulin-induced PKC ζ m-RNA change

was not similar to that in adipocytes of LETO rats. At 10 and 20 min post stimulation with 10 nM insulin the second peak of DG generation was decreased in adipocytes of OLETF rats at 18 weeks of age ($P<0.01$ vs values in LETO) (Table 3a). Insulin-induced [^3H]palmitic acid incorporation into DG and DG content in adipocytes of OLETF rats at 18 weeks of age were also decreased compared with those of LETO rats (Table 3b). Diacylglycerol content was measured by the DG-kinase method as shown in Materials and Methods. Insulin-induced DG content in adipocytes of 18-week-old OLETF rats at 2 and 20 min was significantly decreased compared with that of LETO rats (Table 3c).

Insulin- or TPA-induced protein kinase C (PKC) translocation in adipocytes of OLETF and LETO rats

At 12 weeks of age the cytosolic PKC activity was decreased to 38% of the unstimulated level (control) and membrane-associated PKC activity was increased to 198% of control in adipocytes of LETO rats during treatment with 10 nM insulin, which was not different from that of OLETF rats. However, insulin-induced translocation of PKC activity in adipocytes of 18-week-old OLETF rats was markedly reduced compared with that of LETO rats (cytosolic PKC activity during treatment with 10 nM insulin: LETO $39 \pm 14\%$ vs OLETF $68 \pm 18\%$, $P<0.05$; membrane-associated PKC activity during treatment with 10 nM insulin: LETO 258 ± 32 vs OLETF $130 \pm 35\%$, $P<0.05$) (Fig. 3).

Immunoreactive analysis of PKC β indicated that insulin-induced PKC β translocation in adipocytes of OLETF rats was slightly smaller than that of LETO rats at 12 weeks of age. Densitometric analysis of changes in PKC β immunoreactivity in adipocytes of OLETF rats was similar to that of LETO rats during treatment with 10 nM insulin. TPA-induced PKC translocation was not different in adipocytes of OLETF and LETO rats at 12 weeks of age (data not shown). At 18 weeks of age, membrane-associated PKC β immunoreactivity in adipocytes of OLETF rats was slightly higher than that of LETO rats before insulin stimulation, but insulin-stimulated increases of membrane-associated and decreases of cytosolic PKC β immunoreactivities in adipocytes of OLETF rats were smaller compared with LETO rats as shown in Fig. 4a. PKC β immunoreactivity in adipocytes of OLETF rats was

Figure 7 Insulin- and TPA-stimulated (a) PI 3-kinase activity and (b) p85 subunit of PI 3-kinase immunoreactivity in adipocytes of OLETF and LETO rats. PI 3-kinase activity and p85 subunit of PI 3-kinase immunoreactivity were measured as described in Materials and Methods. (c) Densitometric data of PI 3-kinase activity and p85 subunit of PI 3-kinase immunoreactivity in adipocytes of OLETF (solid bars) and LETO (open bars) rats. Results are means $\pm$ s.e. of three separate experiments. ** $P<0.01$, * $P<0.05$ vs OLETF rats.

also smaller than that of LETO rats by densitometric analysis (Fig. 4b) (cytosolic fraction in adipocytes of OLETF vs LETO rats: 0 min 100%, 5 min $96 \pm 8\%$ vs $53 \pm 7\%$, $P < 0.02$, 10 min $94 \pm 9\%$ vs $65 \pm 6\%$, 20 min $89 \pm 8\%$ vs $60 \pm 7\%$; membrane fraction in OLETF vs LETO adipocytes: 0 min 100%, 5 min $111 \pm 11\%$ vs $144 \pm 12\%$, 10 min $121 \pm 13\%$ vs $235 \pm 18\%$, $P < 0.01$, 20 min $108 \pm 10\%$ vs $230 \pm 19\%$, $P < 0.01$). These results indicated that insulin-induced PKC β translocation in adipocytes of OLETF rats was markedly decreased compared with that of LETO rats. TPA-induced PKC β translocation in adipocytes of OLETF rats was also decreased as indicated in Fig. 4b. Densitometric analysis of PKC β immunoreactivity in adipocytes of OLETF and LETO rats revealed significantly decreased TPA-induced PKC β translocation in adipocytes of OLETF rats (cytosolic fraction in adipocytes of OLETF vs LETO rats: 0 min 100%, 5 min $91 \pm 9\%$ vs $88 \pm 8\%$, 10 min $85 \pm 7\%$ vs $40 \pm 6\%$, $P < 0.01$, 20 min $83 \pm 9\%$ vs $48 \pm 7\%$, $P < 0.05$; membrane fraction in adipocytes of OLETF vs LETO rats: 0 min 100%, 5 min $104 \pm 9\%$ vs $220 \pm 15\%$, $P < 0.01$, 10 min $125 \pm 13\%$ vs $160 \pm 12\%$, 20 min $132 \pm 12\%$ vs $170 \pm 10\%$).

Immunoblot analysis of insulin-induced translocation of protein kinase C β I, β II, ϵ and ζ in adipocytes of OLETF and LETO rats

Analysis of PKC β I, β II, ϵ and ζ isoforms in adipocytes of OLETF and LETO rats at 18 weeks of age by immunoblot indicated weak insulin-induced translocations of PKC β I, β II, ϵ and ζ from cytosol to membrane in OLETF rats compared with those in LETO rats; increases of each membrane-associated PKC isoform by insulin in adipocytes of OLETF rats were smaller than those of LETO rats (Fig. 5a). Densitometric analyses of PKC β I, β II, ϵ and ζ isoforms showed that membrane-associated PKC β I at 20 min, PKC ϵ at 10 and 20 min and PKC β II and PKC ϵ at 10, 20, 60, 120, 180 and 240 min after treatment with insulin in adipocytes of OLETF rats were significantly higher than those of LETO rats, but insulin-induced alterations of cytosolic PKC β I, β II, ϵ and ζ in adipocytes of OLETF rats were not significantly different from those of LETO rats as shown in Fig. 5b.

Alterations of insulin-induced PKC β I, β II, ϵ , and ζ mRNA levels in adipocytes of OLETF and LETO rats

Basal levels of insulin-induced PKC β I, β II, ϵ , and ζ mRNA in adipocytes of OLETF rats were lower than those in LETO rats and, moreover, alterations of each insulin-regulated PKC isoform in adipocytes of OLETF rats were slower than those in LETO rats (Fig. 6a). Densitometric analyses of PKC isoform mRNA showed that the insulin-regulated decrease in PKC β I mRNA in OLETF rats was not significantly different from that

in LETO rats, while the insulin-regulated increases in PKC β II, ϵ , and ζ mRNA in OLETF rats were slower than those in LETO rats. The insulin-mediated first peak of PKC β II mRNA at 10 min was diminished in OLETF rats. Insulin-mediated peaks of PKC ϵ and ζ mRNA in OLETF rats were slower than those in LETO rats (Fig. 6c).

Alterations of insulin- and TPA-induced PI 3-kinase activity and p85 subunit of PI 3-kinase immunoreactivity in adipocytes of OLETF and LETO rats

Insulin- and TPA-induced increases in PI 3-kinase activity and p85 subunit of PI 3-kinase immunoreactivity in adipocytes of OLETF rats were suppressed compared with those of LETO rats (Fig. 7a,b). Densitometric analyses of the p85 subunit of PI 3-kinase (upper 2 panels) and PI 3-kinase activity (lower 2 panels) showed that insulin- and TPA-induced increases in p85 immunoreactivity and PI 3-kinase activity in LETO rats were significantly higher than those in OLETF rats as shown in Fig. 7c.

Discussion

Previous reports of impaired PKC activity from obese Zucker rats heart and liver and a decrease in the responsiveness of glucose uptake to insulin and TPA in Zucker and old-obese rats (Ishizuka *et al.* 1993, Van de Werve *et al.* 1987) prompted us to investigate PKC β immunoreactivity and PKC activity in OLETF rats.

Insulin resistance occurs in adipocytes and skeletal muscle in OLETF rats (Kawano *et al.* 1992). Insulin binding is also decreased in obese Zucker rats (King *et al.* 1992). Several investigators have reported that glucose transporter levels are maintained in insulin-resistant soleus muscle (Crettaz *et al.* 1980, Friedman *et al.* 1990, King *et al.* 1992), but the translocation of glucose transporter depends on a number of cellular factors, including PKC, as reported in adipocytes and soleus muscle (Obermaier-Kusser *et al.* 1989, Cleland *et al.* 1990). As we have shown previously, PKC activity is lower in soleus muscle of the old-obese rats (Ishizuka *et al.* 1993) and Zucker obese rats (Cooper *et al.* 1993). However, since PKC activity did not exhibit much difference in adipocytes (Ishizuka *et al.* 1993), we could not attribute the decreased glucose transport activity simply to the decreased basal PKC activity in adipocytes. Recently, insulin binding, receptor autophosphorylation and the tyrosine kinase activity of partially purified insulin receptor from skeletal muscles of OLETF rats have been shown not to differ from those of LETO rats (Sato *et al.* 1994). In the present study, we have shown reduced activation and translocation of PKC β by insulin and TPA in adipocytes of OLETF rats.

There are several explanations for the decreases in PKC activation and translocation by insulin and TPA in OLETF rat adipocytes. First, the decreased PKC activation

resulting from decreased generation of DG by insulin could decrease the translocation of PKC. In fact, as indicated in Fig. 3, insulin-induced DG generation, especially the second peak of DG, was reduced and subsequently the insulin-provoked PKC translocation was also decreased.

Secondly, the synthesis of new PKC is decreased due to defective translation or decreased transcription of PKC gene or a genetic mutation yielding an inactive or altered PKC enzyme. In either case, a decreased activation of PKC may explain alterations in glucose transport activity which result in insulin resistance in OLETF rats. In previous papers, we have demonstrated in rat adipocytes that down-regulation of PKC by chronic treatment by phorbol esters or antisense oligonucleotides directed against PKC α and β suppressed insulin-stimulated glucose uptake (Ishizuka *et al.* 1991, Cooper *et al.* 1992, Farese *et al.* 1992). In adipocytes of OLETF rats at 18 weeks of age, high glucose and hyperinsulinemia, as shown in Table 2, may result in down-regulation of PKC mRNA, especially the PKC β II isoform with subsequently reduced insulin-induced activation of PKC. In addition to decreased basal PKC β II mRNA expression, insulin-regulated alterations of PKC isoform mRNA levels in adipocytes of OLETF rats were slower than those of LETO rats as indicated in Fig. 6. Recently, it has been reported that insulin regulates alternative splicing of PKC β , and that switching PKC β I to PKC β II may provoke long activation of protein kinase C because of down-regulation-resistant PKC β II (Chalfant *et al.* 1995). Thus, delayed and weak alternative splicing of PKC β and alteration of PKC isoforms may contribute to weak activation of PKC isoforms, and subsequently cause diminished glucose uptake in adipocytes of OLETF rats.

Activation of insulin-induced insulin-receptor substrate-1 (IRS-1) and PI 3-kinase by insulin or TPA was also examined. Although basal p85 subunit of PI 3-kinase immunoreactivity in adipocytes of OLETF rats was slightly higher than that in LETO rats, insulin-stimulated PI 3-kinase activity in adipocytes of OLETF rats was decreased compared with that in adipocytes of LETO rats. Unexpectedly, phorbol ester-stimulated PI 3-kinase activation was observed in adipocytes of OLETF and LETO rats. Interestingly, like insulin-stimulated activity, TPA-stimulated PI 3-kinase activity was also decreased in adipocytes of OLETF rats compared with those of LETO rats. It has been reported that PI 3-kinase activation by PKC stimulation could be observed in human platelets (King *et al.* 1991). Accordingly, phorbol ester may phosphorylate pleckstrin homology which is contained in IRS-1 (Voliovitch *et al.* 1995), and subsequently PI 3-kinase activity may be provoked through IRS-1 phosphorylation of pleckstrin homology. Finally, weak PKC signaling may cause decreased PI 3-kinase activation and a subsequent insulin-induced decreased metabolic effect.

In summary, hyperglycemia and/or hyperinsulinemia activate and eventually down-regulate PKC, and suppress activation of PKC due to a defective response of insulin-induced DG generation. This decreased PKC activation may induce the deterioration of insulin-induced glucose uptake in rat adipocytes.

Further studies will enable us to determine whether the decreased PKC activation is the result of a genetic or acquired defect in PKC gene expression, which results in insulin resistance in NIDDM.

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