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Am J Physiol Endocrinol Metab 276:196-204, 1999.

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DHEA improves glucose uptake via activations of protein kinase C and phosphatidylinositol 3-kinase

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Ishizuka, Tatsuo, Kazuo Kajita, Atsushi Miura, Masayoshi Ishizawa, Yoshinori Kano, Satomi Itaya, Mika Kimura, Naoya Muto, Tomoatsu Mune, Hiroaki Morita, and Keigo Yasuda. DHEA improves glucose uptake via activations of protein kinase C and phosphatidylinositol 3-kinase. *Am. J. Physiol.* 276 (*Endocrinol. Metab.* 39): E196–E204, 1999.—We have examined the effect of adrenal androgen, dehydroepiandrosterone (DHEA), on glucose uptake, phosphatidylinositol (PI) 3-kinase, and protein kinase C (PKC) activity in rat adipocytes. DHEA (1 μ M) provoked a twofold increase in 2-[3 H]deoxyglucose (DG) uptake for 30 min. Pretreatment with DHEA increased insulin-induced 2-[3 H]DG uptake without alterations of insulin specific binding and autophosphorylation of insulin receptor. DHEA also stimulated PI 3-kinase activity. [3 H]DHEA bound to purified PKC containing PKC- α , - β , and - γ . DHEA provoked the translocation of PKC- β and - ζ from the cytosol to the membrane in rat adipocytes. These results suggest that DHEA stimulates both PI 3-kinase and PKCs and subsequently stimulates glucose uptake. Moreover, to clarify the in vivo effect of DHEA on Goto-Kakizaki (GK) and Otsuka Long-Evans fatty (OLETF) rats, animal models of non-insulin-dependent diabetes mellitus (NIDDM) were treated with 0.4% DHEA for 2 wk. Insulin- and 12-*O*-tetradecanoyl phorbol-13-acetate-induced 2-[3 H]DG uptakes of adipocytes were significantly increased, but there was no significant increase in the soleus muscles in DHEA-treated GK/Wistar or OLETF/Long-Evans Tokushima (LETO) rats when compared with untreated GK/Wistar or OLETF/LETO rats. These results indicate that in vivo DHEA treatment can result in increased insulin-induced glucose uptake in two different NIDDM rat models.

dehydroepiandrosterone; non-insulin-dependent diabetes mellitus; 2-deoxyglucose

GLUCOCORTICOID EXCESS causes insulin resistance. However, the mechanisms involved are unknown (1). Studies have shown that glucocorticoids affect specific insulin binding (27), tyrosine kinase activity (20, 35), and/or glucose transporter (7). Recently, dose-response inhibition of insulin-induced 2-[3 H]deoxyglucose (2-DG) uptake by dexamethasone and prednisolone in rat adipocytes has been observed, and glucocorticoids have been shown to stimulate the protein kinase C (PKC) isoform via binding to the regulatory subunit of PKC (17, 18). On the other hand, adrenal androgen, dehydroepiandrosterone (DHEA; 3 β -hydroxy-5-androsten-17-one),

and its sulfate derivative are found in abundance in the human (28), although their physiological roles are still unknown. Serum concentrations of DHEA in 60-yr-old men show a gradual decrease compared with young men aged 25–30 yr. This decrease occurs as the incidence of atherosclerosis, obesity, and diabetes increases, suggesting that administration of DHEA may protect against the development of these disorders (2). In genetically diabetic (*db/db*) mice, DHEA administration prevents the development of diabetes mellitus (5). Recently, it has been reported that DHEA treatment reduces fat accumulation and protects against insulin resistance via an increase in phosphatidylinositol (PI) 3-kinase after immunoprecipitation with insulin receptor substrate-1 (IRS-1) in male rats (10). We have investigated the effects of DHEA on insulin-induced glucose uptake in vivo and in vitro in Wistar, Goto-Kakizaki (GK; see Refs. 8 and 9), and Otsuka Long-Evans fatty (OLETF) rat (16, 21) adipocytes.

MATERIALS AND METHODS

Materials. Pork insulin was obtained from Novo (Copenhagen, Denmark). 2-[1,2- 3 H]DG was purchased from New England Nuclear (Boston, MA). Phenylmethylsulfonyl fluoride (PMSF), leupeptin, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), BSA, phosphatidylserine, dioleoin, histone (type III-S), D-glucose, ATP, dexamethasone, prednisolone, and DHEA were purchased from Sigma (St. Louis, MO). Silicone oil was obtained from Aldrich Chemical (Milwaukee, WI). [γ - 32 P]ATP (3,000 Ci/mmol), 2-[1,2- 3 H]DG (50 Ci/mmol), L-[1- 14 C]glucose (47 mCi/mmol), [6,7- 3 H]dexamethasone (50 Ci/mmol), and [1,2- 3 H]DHEA (60 Ci/mmol) were purchased from New England Nuclear (Tokyo, Japan). RU-38486 (17 β hydroxy-11 β ,4-dimethylaminophenyl-17 α -propynyl estra 4,9 diene-3-one) was generously donated by the Roussel-Uclaf Research Center. All other chemicals were of reagent grade or better. Polypropylene plastic tubes were used in each experiment, unless otherwise stated.

Adipocyte experiments. Male Wistar and GK rats, a non-insulin-dependent diabetes mellitus (NIDDM) animal model (8, 9) weighing 150–200 g (8 wk of age), and Long-Evans Tokushima (LETO, control) and OLETF rats (16 wk of age; see Refs. 16, 21) were fed with CE2 (Japan Clea, Tokyo, Japan) ad libitum, treated with CE2 powder containing 0.4% DHEA for 2 wk, and then killed by decapitation. As shown in Table 1, in vivo treatment with 0.4% DHEA for 2 wk resulted in significant increases in plasma DHEA concentration in GK/Wistar and OLETF/LETO rats. Isolated adipocytes were obtained by collagenase digestion of rat epididymal fat pads (26, 29) in Krebs-Ringer phosphate (KRP) buffer (pH 7.4) containing 127 mM NaCl, 12.3 mM NaH₂PO₄, 5.1 mM KCl, 1.3 mM MgSO₄, 1.4 mM CaCl₂, 3% BSA, and 2.5 mM glucose. Adipocytes were washed two times, preincubated at 37°C in glucose-free KRP buffer containing 1% BSA for 30 min, and then incubated with or without (control) 10⁻⁶ M DHEA

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Table 1. DHEA concentration in GK/Wistar and OLETF/LETO rats after treatment with 0.4% DHEA for 2 wk

| | n | DHEA Concentration, nM | |
|--------|----|------------------------|--------------|
| | | Untreated | DHEA treated |
| GK | 10 | 16 ± 1 | 71 ± 2* |
| Wistar | 10 | 10 ± 1 | 70 ± 1* |
| OLETF | 15 | 10 ± 4 | 94 ± 3* |
| LETO | 15 | 7 ± 2 | 45 ± 18* |

Values are means ± SE; n, no. of rats. DHEA, dehydroepiandrosterone; GK, Goto-Kakizaki; OLETF, Otsuka Long-Evans fatty; LETO, Long-Evans Tokushima. *P < 0.01 vs. untreated values by ANOVA.

(dissolved in <0.01% ethanol) for 60 min, followed by incubation with 10 nM insulin for 30 min. There was no effect of 0.01% ethanol, used for the control, on insulin-induced glucose uptake in rat adipocytes. 2-[³H]DG (0.08 μCi) and unlabeled 2-DG (0.05 mM) were then added to 300 μl of a 10% (vol/vol) adipocyte suspension, and uptake of 2-[³H]DG was measured over 1 min. Corrections for trapped buffer or non-carrier-mediated uptake were determined by measuring uptake in the presence of 70 μM cytochalasin, which reduced control and agonist-stimulated values (14).

PKC experiments. Adipocytes were incubated with or without (control, 0 min) 1 μM DHEA for 5, 10, and 20 min. Reactions were terminated by the addition of 20 mM Tris·HCl buffer (pH 7.5) containing 0.25 M sucrose, 1.2 mM EGTA, 0.1 mM PMSF, 20 μg/ml leupeptin, and 20 mM 2-mercaptoethanol (*buffer I*), washed two times, and homogenized in *buffer I*. The homogenates were centrifuged for 60 min at 105,000 g to obtain the cytosol and membrane fractions. The latter was homogenized in *buffer I* containing 5 mM EGTA, 2 mM EDTA, and 1% Triton X-100. Activation of PKC in rat adipocytes was assayed by changes in the subcellular distribution of immunoreactive PKC with methods described previously (14, 15). Equal amounts of cytosol or membrane-associated fraction were prepared, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and incubated first with polyclonal antiserum raised to synthetic peptide to PKC-β and -ζ (GIBCO) and second with goat anti-rabbit globulin complexed to alkaline phosphatase (Sigma). This immunoblotting method detected a single major immunoreactive band that comigrated on SDS-PAGE and blotted identically with 80-kDa (PKC-β) and 73-kDa (PKC-ζ) synthetic peptides.

Soleus muscle experiments. Soleus muscles were excised, and muscle tension was maintained by ligatures attached to the tendons. The two solei from each rat provided one control and one stimulated sample. Soleus muscles were incubated in 25-ml Erlenmeyer flasks under 95% O₂-5% CO₂ in 5 ml Krebs-Ringer bicarbonate (KRB) buffer (119 mM NaCl, 4.8 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 1 mM CaCl₂, 24 mM NaHCO₃, 12 mM HEPES, 0.1% BSA, and 2 mM sodium pyruvate). For studies of glucose transport, after an initial 30-min incubation in KRB buffer containing 5 mM glucose, the solei were washed and incubated for 30 min in glucose-free KRB for 30 min with or without insulin. 2-[³H]DG (1 μCi), L-[1-¹⁴C]glucose (0.1 μCi), and 0.1 mM unlabeled 2-DG were then added, and incubation was continued for 10 min. After incubation, tissue was removed, rapidly rinsed in isotope-free medium, blotted, weighed, homogenized in 5% trichloroacetic acid, and counted simultaneously for ³H and ¹⁴C. Corrections for 2-[³H]DG in tissue samples unrelated to specific transport were determined by measurement of radioactivity of

L-[¹⁴C]glucose. As reported previously (15), uptake of 2-[³H]DG was linear for at least 30 min.

DHEA binding study. For [³H]DHEA specific binding to purified PKC from rat brain, Mono Q column-purified PKC was obtained using an HPLC system as described previously (14, 15), and then the collected samples were concentrated fivefold in *buffer I* containing 5 mM EGTA, 2 mM EDTA, and 5% glycerol. Moreover, purified PKC (BIOML Research Laboratories), 50 nM [³H]DHEA, 1,000-fold DHEA, and 100-fold RU-38486 were incubated for 24 h at 4°C with and without 0.5 mM Ca²⁺ and were separated by the addition of charcoal dextran. Radioactivity in the supernatant was measured with a liquid scintillation counter. [³H]DHEA specific binding to free adipocytes was carried out as described previously (34). Briefly, free adipocytes (80 μl) and 50 nM [³H]DHEA 1,000-fold unlabeled DHEA in the presence or absence of 100-fold RU-38486 were incubated for 60 min at 37°C, separated by passage through Whatman filter paper (25 mm), and rinsed several times with an excess of 0.9% saline. The radioactivity in this paper was counted with a liquid scintillation counter.

PI 3-kinase assay. Isolated adipocytes were treated with or without insulin (10 nM) for 0, 1, 5, 10, and 60 min at 37°C, lysed in buffer containing 1% (vol/vol) Nonidet P-40 (NP-40; see Ref. 11), and immunoprecipitated with an anti-phosphotyrosine antibody (19) and protein A-agarose. The immunoprecipitates were washed and subjected to the PI 3-kinase assay as described elsewhere (11). Briefly, cells were treated with 10 nM insulin, 1 μM TPA, or 1 μM DHEA for the indicated periods, lysed in the buffer (20 mM Tris·HCl, pH 7.4, 1% NP-40, 0.5 mM EDTA, 5% glycerol, 1 mM orthovanadate, and 20 μM *p*-amidinophenylmethanesulfonyl fluoride hydrochloride), and sonicated. The homogenates were centrifuged at 15,000 rpm for 20 min, and the indicated supernatants were incubated with 4 μg of anti-phosphotyrosine antibody for 2 h at 4°C. The immunocomplexes were precipitated with 40 μl of protein A-agarose and washed with NP-40-free buffer (10 mM Tris·HCl, pH 7.4, 100 mM NaCl, and 1 mM dithiothreitol) two times. The immunoprecipitates were subjected to the PI

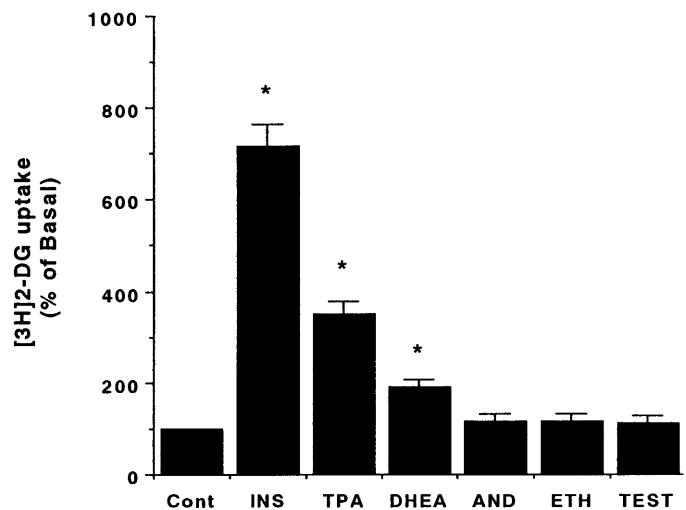


Fig. 1. Agonists stimulate 2-[³H]deoxyglucose (2-[³H]DG) uptake in rat adipocytes. Isolated adipocytes (10% cell suspension) were stimulated with or without (control, Cont) 10 nM insulin (INS), 1 μM phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), 1 μM dehydroepiandrosterone (DHEA), androstenedione (AND), ethiocholanolone (ETH), or 1 μM testosterone (TEST) for 30 min. 2-[³H]DG (0.05 mM, 0.08 μCi) was added to 300 μl of a 10% adipocyte suspension, and then 2-[³H]DG uptake was measured. Control value was set at 100%. Data are plotted as means ± SE of 6 separate experiments. *P < 0.01, INS or TPA vs. Cont.

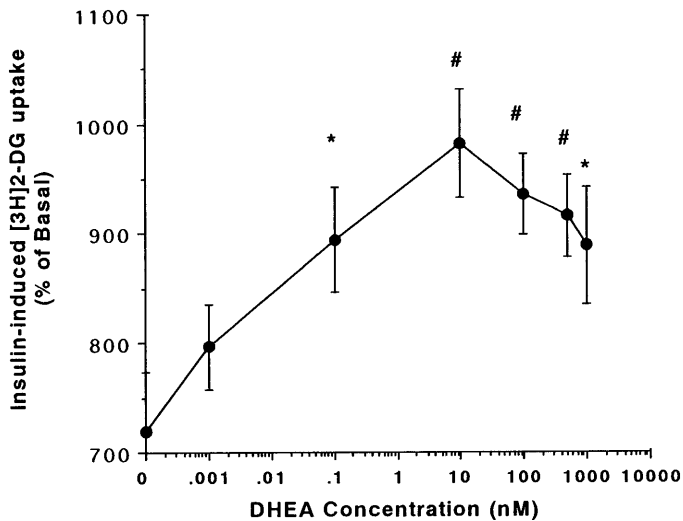


Fig. 2. Effect of DHEA on insulin-induced 2- ^3H]DG uptake in rat adipocytes. Isolated adipocytes were stimulated with 10 nM insulin after pretreatment with 1 μM DHEA for 60 min, and then glucose uptake was measured as shown in Fig. 1 legend. Data are plotted as the means \pm SE of 5 separate experiments. * $P < 0.01$ and # $P < 0.05$ by Fisher's protected least significant difference (PLSD) test.

3-kinase assay in a 50- μl reaction mixture containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl_2 , 0.5 mM EGTA, 100 μM PI, 100 μM phosphatidylserine, and 10 μM [γ - ^{32}P]ATP (0.1 $\mu\text{Ci}/\mu\text{l}$). After 10 min at 30°C, the reaction was stopped by adding 200 μl of 1 M HCl and 80 μl of chloroform-methanol (1:1, vol/vol). A 30- μl portion of the lower layer was spotted on a Silica Gel 60 plate (Merck) and was developed in chloroform-methanol-25% NH_4Cl -water (43:38:5:7, vol/vol/vol/vol). The radioactive PI phosphate spot was detected by autoradiography, and scrapes from the plate were counted by liquid scintillation counting.

Immunoblot. Immunoprecipitate (500 μg protein) with an anti-phosphotyrosine antibody (5 μg) or anti-insulin receptor antibody (5 μg ; Upstate Biotechnology, Lake Placid, NY) was separated by SDS-PAGE and transferred to nitrocellulose paper. The paper was blocked with 3% gelatin Tris-buffered saline and was incubated with an anti-p85 PI 3-kinase antibody or anti-phosphotyrosine antibody (1:1,000 dilution; Transduction Laboratories, Lexington, KY) for 4–5 days. Protein bands were located with an enhanced chemiluminescence system (Amersham, Tokyo, Japan). Immunoblots were quantified by laser scanning densitometry.

Insulin binding studies. Isolated adipocytes were suspended in KRP buffer, incubated for 60 min with or without (control) 1 μM DHEA, and then incubated with [^{125}I]insulin

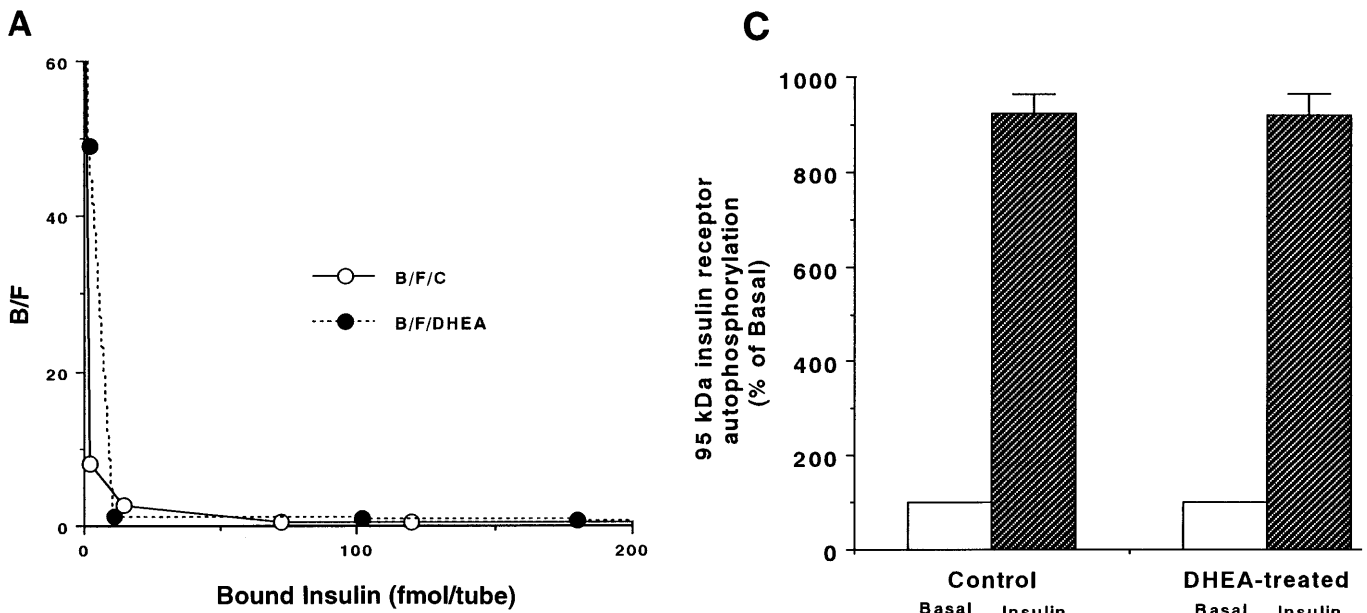


Fig. 3. Effect of DHEA on insulin specific binding. Isolated adipocytes were suspended in Krebs-Ringer phosphate buffer and incubated for 60 min with or without (control) 1 μM DHEA and then with [^{125}I]insulin (2,000 Ci/mmol) and unlabeled insulin (1–1,000 nM) in plastic tubes at 25°C in a shaking water bath for 60 min. Cells were then removed using the addition of silicone oil, and radioactivity was determined. Data are plotted by Scatchard analysis as the mean of triplicate determinations (A). Immunoprecipitates with antibody against insulin receptor were separated by SDS-PAGE. Immunoblot analysis of 100 nM insulin-induced 95-kDa tyrosine autophosphorylation of insulin receptor was performed in DHEA-treated cells for 60 min and control cells. Representative experiment is shown (B), and data by densitometric analysis are plotted as the means \pm SE of 3 separate experiments (C). B/F, bound-to-free ratio; C, control.

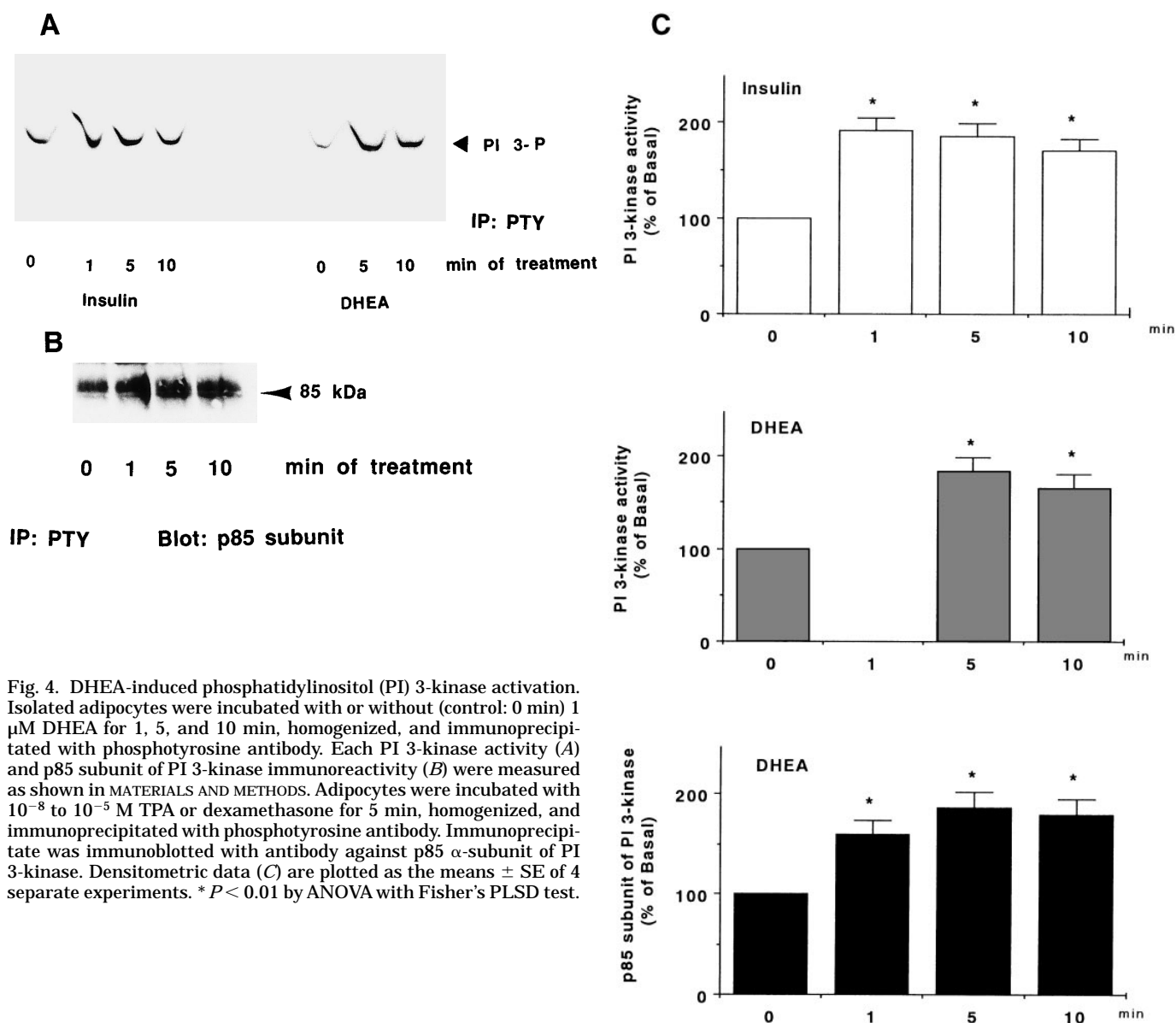


Fig. 4. DHEA-induced phosphatidylinositol (PI) 3-kinase activation. Isolated adipocytes were incubated with or without (control: 0 min) 1 μ M DHEA for 1, 5, and 10 min, homogenized, and immunoprecipitated with phosphotyrosine antibody. Each PI 3-kinase activity (A) and p85 subunit of PI 3-kinase immunoreactivity (B) were measured as shown in MATERIALS AND METHODS. Adipocytes were incubated with 10^{-8} to 10^{-5} M TPA or dexamethasone for 5 min, homogenized, and immunoprecipitated with phosphotyrosine antibody. Immunoprecipitate was immunoblotted with antibody against p85 α -subunit of PI 3-kinase. Densitometric data (C) are plotted as the means \pm SE of 4 separate experiments. * $P < 0.01$ by ANOVA with Fisher's PLSD test.

(2,000 Ci/mmol; Amersham) and unlabeled insulin (1–1,000 nM) in plastic tubes at 25°C in a shaking water bath for 60 min as described previously (17). Incubations were terminated by removing 300- μ l aliquots from the cells in plastic microfuge tubes to which 100 μ l silicone oil had been added. The cells were then separated by cutting a plastic tube just over the silicone oil layer, and the radioactivity was determined. All studies were performed in triplicate.

Incorporation of ^3H in diacylglycerol. Adipocytes were incubated for 30 min in 0.5 ml KRP buffer. [^3H]glycerol (10 μ Ci) was then added, and, after prelabeling adipocytes for 15 min, a vehicle (control) or 10 nM insulin was added after pretreatment with 1 μ M DHEA for 60 min. Incubation was continued for 2, 5, 10, and 20 min. 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 (6, 12).

Statistical analysis. Statistical comparisons were performed using ANOVA followed by Fisher's protected least significant difference (PLSD) test. Unless otherwise stated, all data are expressed as the means \pm SE.

RESULTS

Effect of DHEA on insulin-induced 2- ^3H]DG uptake. DHEA (1 μ M) alone stimulated 2- ^3H]DG uptake by 150–200% from the basal level within 30 min ($P < 0.05$ by ANOVA with Fisher's PLSD) but did not stimulate androstenedione, ethiocholanolone, or testosterone (Fig. 1). Maximal effect of DHEA on glucose uptake was observed at 1 μ M (data not shown). Pretreatment with 1 μ M DHEA resulted in a significant increase ($P < 0.01$ by ANOVA with Fisher's PLSD) in insulin-induced 2- ^3H]DG uptake. Maximal effect of DHEA on insulin-induced 2- ^3H]DG uptake was observed at 10^{-8} M (Fig. 2).

Effect of DHEA on insulin specific binding to its receptor and 95-kDa autophosphorylation of insulin receptor. To clarify the effect of DHEA on insulin specific binding activity and insulin receptor autophosphorylation activity, which is the first insulin signaling step, we examined [^{125}I]insulin specific binding to the

receptor of adipocytes and 95-kDa autophosphorylation of the insulin receptor β -subunit. As shown in Fig. 3, there was no significant difference in [125 I]insulin specific binding to the receptor of adipocytes (control cells bound $2.9 \pm 0.2\%$ of [125 I]insulin/tube, cells treated with DHEA bound $2.8 \pm 0.3\%$ of [125 I]insulin/tube; Fig. 3A), and there was also no significant difference in 95-kDa insulin receptor autophosphorylation (Fig. 3B) between treatment with and without $1 \mu\text{M}$ DHEA. Densitometric analysis indicated that there was no difference in insulin-induced 95-kDa autophosphorylation between DHEA-treated and untreated adipocytes (925 ± 37 vs. $918 \pm 46\%$; Fig. 3C).

DHEA-induced PI 3-kinase activation. Moreover, to clarify the effect of DHEA on PI 3-kinase, which binds to tyrosine-phosphorylated IRS-1 via the SH2 domain of PI 3-kinase, downstream of the insulin signaling pathway, we examined whether DHEA stimulates PI 3-kinase in rat adipocytes. When adipocytes were incubated with $1 \mu\text{M}$ DHEA, unexpectedly, enzyme activity (Fig. 4A) and the p85 subunit of PI 3-kinase (Fig. 4B), after immunoprecipitation with anti-phosphotyrosine antibody, increased for 5 and 10 min, similar to 100 nM insulin-induced PI 3-kinase activation (Fig. 4A, left). Densitometric data are also shown in Fig. 4C.

Effect of DHEA on diacylglycerol production. To resolve the DHEA-stimulated glucose uptake mechanism, we focused on diacylglycerol-PKC signaling. When adipocytes were labeled with [^3H]glycerol, $1 \mu\text{M}$ DHEA alone stimulated diacylglycerol production to 150% from basal levels (Fig. 5A). Insulin-stimulated incorporation of [^3H]glycerol in [^3H]diacylglycerol for 2 and 20 min was markedly enhanced almost twofold by pretreatment with $1 \mu\text{M}$ DHEA for 60 min (Fig. 5B).

DHEA-induced PKC translocation. DHEA stimulated both PI 3-kinase activation and diacylglycerol production as shown in Figs. 4 and 5. We examined whether DHEA activates PKC- ζ , which is thought to be downstream of PI 3-kinase (23), and PKC- β . Isolated adipocytes were treated with or without (control: 0 min) $1 \mu\text{M}$ DHEA for 5, 10, and 20 min. Cytosolic PKC- β and - ζ immunoreactivities gradually decreased, and membrane-associated PKC- β and - ζ immunoreactivity was inversely increased (Fig. 6). Dexamethasone induced PKC- β and - ζ translocations from the cytosol to the membrane, as shown previously (18).

[^3H]DHEA binding to PKC purified from rat brain. When 10 ng purified conventional PKC (PKC- α , - β , and - γ) activity was measured using histone III-s as the substrate in the presence of $0.001\text{--}1 \text{ mM}$ Ca^{2+} , phosphatidylserine, and diolein, the most effective Ca^{2+} concentration was 0.5 mM . On the other hand, in the presence of $40 \mu\text{g/ml}$ phosphatidylserine, protein kinase activity was most activated by the addition of $1 \mu\text{M}$ DHEA in 0.5 mM Ca^{2+} (Fig. 7). Immunoprecipitate with anti-PKC- ζ antibody was also activated in the presence of phosphatidylserine/diolein (data not shown). In addition to the above results, we examined [^3H]DHEA specific binding to conventional PKC and atypical PKC. [^3H]DHEA specific binding to conventional PKC was found to be as shown in Fig. 8. Glucocorticoid receptor antagonist RU-38486 did not affect [^3H]DHEA binding to PKC in

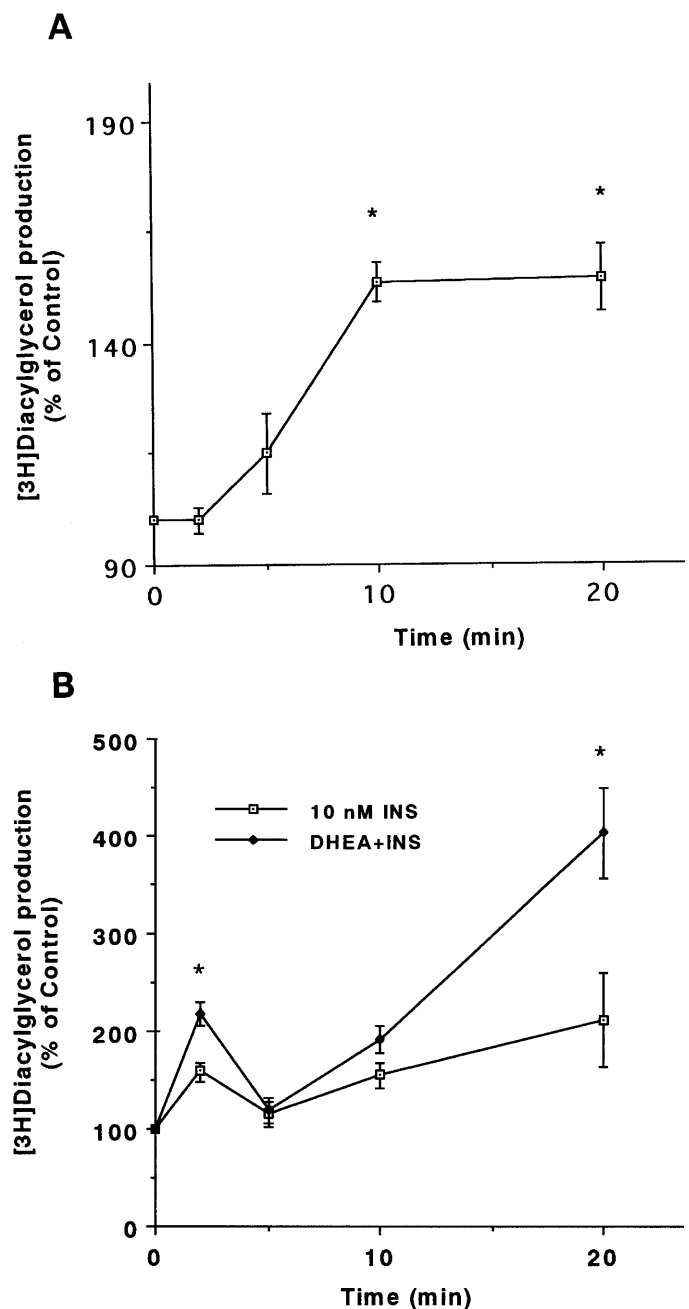


Fig. 5. Effect of DHEA on insulin-induced diacylglycerol production. Isolated adipocytes were prelabeled for 15 min with [^3H]glycerol. Labeled adipocytes were stimulated with $1 \mu\text{M}$ DHEA alone for 2, 5, 10, and 20 min (A). After pretreatment with $1 \mu\text{M}$ DHEA for 60 min, labeled adipocytes were stimulated with 10 nM insulin (B). Reaction was terminated with methanol as shown in MATERIALS AND METHODS. Data are plotted as the means \pm SE of 4 separate experiments. * $P < 0.01$ by ANOVA with Fisher's PLSD test.

vitro. [^3H]DHEA specific binding to PKC- ζ was also observed to a similar extent (data not shown).

Effect of DHEA treatment in vivo on insulin- or TPA-induced glucose uptake in adipocytes of GK/Wistar and OLETF/LETO rats. At 10 wk of age, 10 nM insulin- or $1 \mu\text{M}$ TPA-induced 2-[^3H]DG uptake in adipocytes of GK rats decreased by only 20% compared with Wistar rats. There were no significant differences in body weight (DHEA-treated vs. untreated Wistar

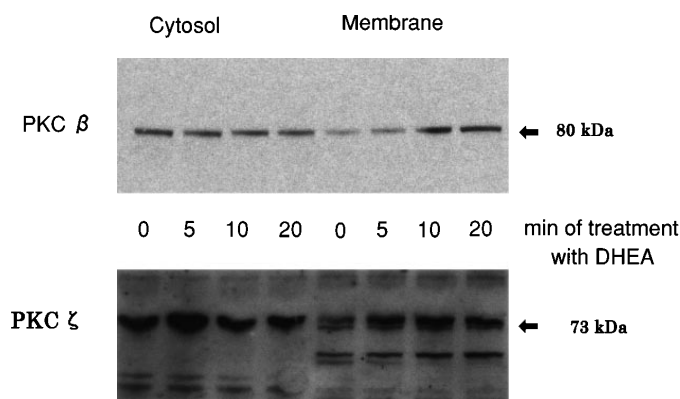


Fig. 6. DHEA-induced protein kinase C (PKC)- β and PKC- ζ translocations. Isolated adipocytes were stimulated with or without (control) 1 μ M DHEA for 5, 10, and 20 min. Cells were homogenized and centrifuged to obtain cytosol and membrane fractions. Each cytosolic and membrane-associated protein (40 μ g) was subjected to SDS-PAGE, transferred to the nitrocellulose membrane, and immunoblotted with PKC- β and PKC- ζ antibodies using enhanced chemiluminescence or alkaline phosphatase procedure.

rats, 270 ± 7 vs. 285 ± 3 g; DHEA-treated vs. untreated GK rats, 252 ± 25 vs. 211 ± 11 g), plasma glucose level (DHEA-treated vs. untreated Wistar rats, 128 ± 14 vs. 130 ± 15 mg/dl; DHEA-treated vs. untreated GK rats, 211 ± 33 vs. 234 ± 14 mg/dl), or plasma insulin level (DHEA-treated vs. untreated Wistar rats, 83 ± 16 vs. 84 ± 18 pM; DHEA-treated vs. untreated GK rats, 79 ± 11 vs. 78 ± 12 pM) in Wistar and GK rats after in vivo treatment with 0.4% DHEA. In the 2-wk treatment with 0.4% DHEA in vivo, 10 nM insulin- or 1 μ M TPA-induced 2-[3 H]DG uptake was significantly increased by 50–100%, but basal 2-[3 H]DG uptake was not increased compared with untreated GK and Wistar rats at 10 wk of age. However, DHEA-induced 2-[3 H]DG uptakes in adipocytes of DHEA-treated GK and Wistar rats were not significantly increased compared with those of untreated GK and Wistar rats (Fig. 9A). It should be noted that glucose uptake in adipocytes of GK rats was not markedly different from that in Wistar

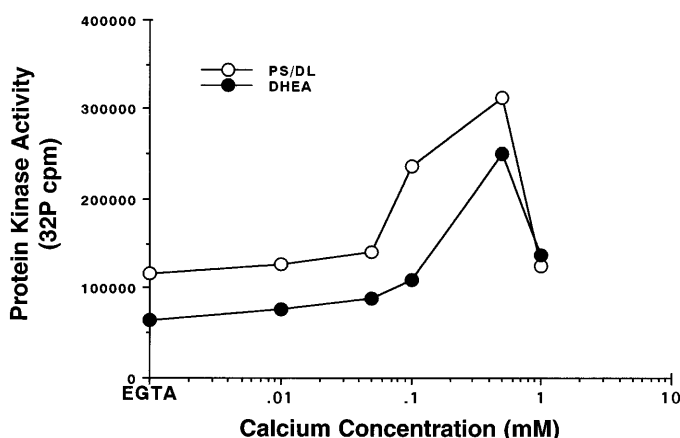


Fig. 7. Ca^{2+} dependence of the ability of DHEA and diolein (DL) with phosphatidylserine (PS) to stimulate purified PKC (BIOML Research Laboratories). Protein kinase activity was assayed by histone phosphorylation activity in the presence of 1 μ M DHEA or 0.4 μ g/ml DL with 40 μ g/ml PS at various concentrations of Ca^{2+} . Data are plotted as the means of 3 separate experiments. cpm, Counts/min.

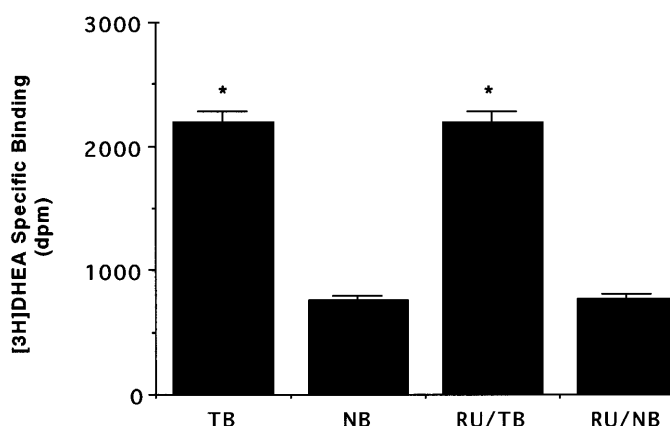


Fig. 8. [3 H]DHEA binding to PKC purified from rat brain. Purified PKC from rat brain (specific activity 1,530 nmol/mg; BIOML Research Laboratories) containing PKC- α , - β , and - γ , [3 H]DHEA, and 1,000-fold cold DHEA were incubated for 24 h at 4°C in the presence or absence of 0.5 mM Ca^{2+} . Total binding, nonspecific binding, total binding in the presence of RU-38486, and nonspecific binding in the presence of RU-38486 are abbreviated as TB, NB, RU/TB, and RU/NB, respectively.

control rats. Therefore, we selected another NIDDM animal model, the OLETF rat, and examined the effect of in vivo DHEA treatment on insulin-, TPA-, or DHEA-induced 2-[3 H]DG uptake. At 10 wk of age, 10 nM insulin- and 1 μ M TPA-induced 2-[3 H]DG uptake in adipocytes of OLETF rats decreased by 45 and 40%, respectively, when compared with LETO rats (control). In the 2-wk treatment with 0.4% DHEA in vivo, there were no significant differences in body weight (DHEA-treated vs. untreated LETO rats, 386 ± 41 vs. 420 ± 45 g; DHEA-treated vs. untreated OLETF, 454 ± 55 vs. 517 ± 58 g) or plasma insulin level (DHEA-treated vs. untreated LETO rats, 182 ± 43 vs. 185 ± 42 pM; DHEA-treated vs. untreated OLETF rats, 257 ± 38 vs. 252 ± 37 pM), but there was a significant difference in plasma glucose of OLETF rats (DHEA-treated vs. untreated LETO rats, 112 ± 25 vs. 124 ± 20 mg/dl; DHEA-treated vs. untreated OLETF rats, 144 ± 19 vs. 201 ± 21 mg/dl; $P < 0.05$ by ANOVA with Fisher's PLSD). After in vivo treatment with DHEA, insulin and TPA-induced glucose uptakes were significantly increased by 50–100% when compared with untreated OLETF and LETO rats, but there was no increase in DHEA-induced glucose uptake (Fig. 9B). These results suggested that DHEA markedly increased glucose transport activity in not only Wistar and LETO rats, but also GK and OLETF rats.

Effect of DHEA treatment in vivo on insulin- or TPA-induced glucose uptake in soleus muscles of OLETF/LETO rats. As shown in Fig. 10, basal and 100 nM insulin- or 1 μ M TPA-stimulated 2-[3 H]DG uptake in soleus muscles of OLETF rats decreased compared with LETO rats ($P < 0.01$ by ANOVA with Fisher's PLSD). However, in the 2-wk treatment with 0.4% DHEA, basal and 100 nM insulin- or 1 μ M TPA-stimulated 2-[3 H]DG uptake values in soleus muscles of DHEA-treated OLETF/LETO rats were not significantly different from those in untreated OLETF/LETO rats.

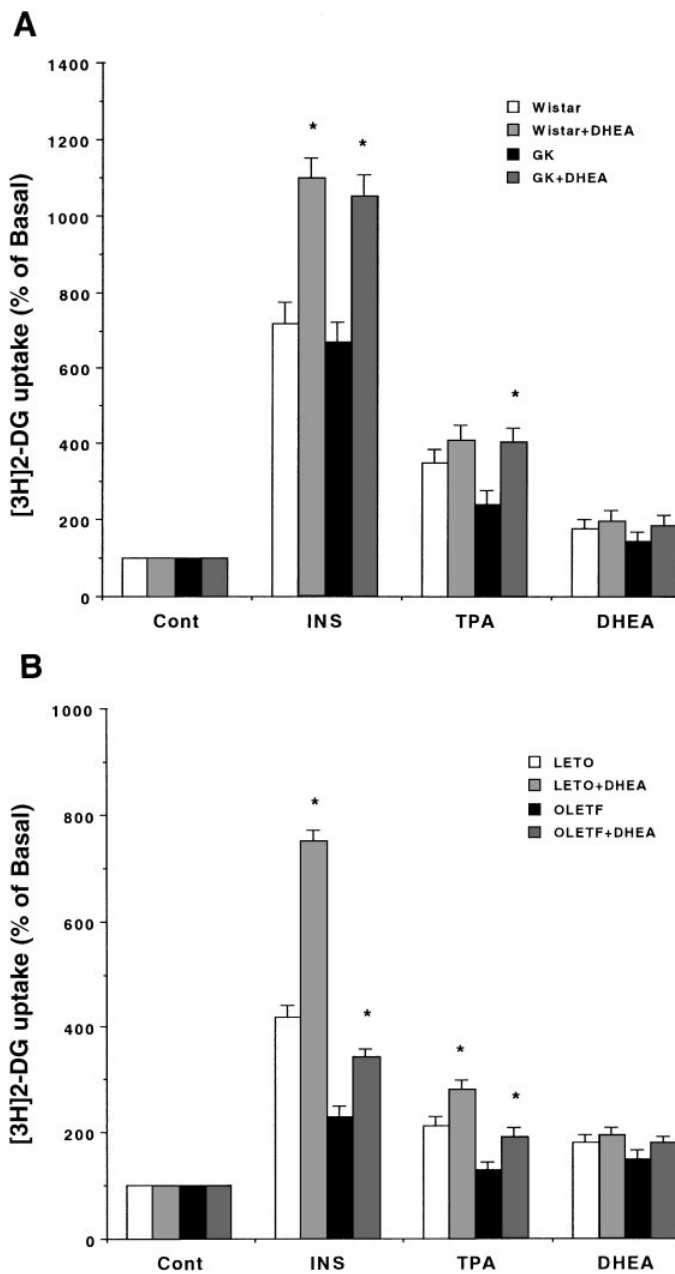


Fig. 9. Effect of in vivo DHEA treatment on insulin- or TPA-stimulated 2- ^3H DG uptake in adipocytes of Goto-Kakizaki (GK)/Wistar and Otsuka Long Evens fatty (OLETF)/Long Evens Tokushima (LETO) rats at 10 and 18 wk of age, respectively. After treatment with 0.4% DHEA in vivo for 2 wk, isolated adipocytes were obtained from epididymal fat pads in GK (A) and OLETF (B) rats. Insulin (10 nM)- and 1 μM TPA-stimulated 2- ^3H DG uptake was measured as shown in MATERIALS AND METHODS in adipocytes of GK and OLETF rats and Wistar and LETO rats at 10 wk of age as controls with or without treatment with DHEA for 2 wk. Data are plotted as means \pm SE of 5 separate experiments. * $P < 0.01$ vs. without DHEA treatment by ANOVA with Fisher's PLSD test.

DISCUSSION

The present findings indicate that DHEA alone stimulates glucose uptake through diacylglycerol-PKC and PI 3-kinase signaling pathways. It has been reported that the glucose uptake mechanism is mediated by an IRS-1-PI 3-kinase pathway (30) and a diacylglycerol-

PKC pathway (15). Recently, the PI 3-kinase inhibitor wortmannin was shown to decrease insulin-induced glucose uptake in rat adipocytes (25). Therefore, PI 3-kinase plays a pivotal role in the regulation of insulin. On the other hand, insulin and phorbol esters increase membrane-associated PKC via increases in diacylglycerol probably via phospholipase C and phospholipase D activation (31). Moreover, atypical PKC- ζ , which is a diacylglycerol-insensitive PKC, operates downstream of PI 3-kinase (23). In addition to the PI 3-kinase-PKC- ζ pathway, PKC- ζ may be a candidate for insulin-induced glucose transport in insulin-sensitive tissue (33). On the basis of insulin signaling, we have found that DHEA alone provokes diacylglycerol production by phospholipase C or phospholipase D activation in rat adipocytes. These results suggest the following mechanisms. First, DHEA binds to a specific membrane receptor. Second, DHEA binds to PKC, leading to subsequent stimulation of phospholipase D. The first suggestion has already been reported as follows (22, 24). Meikle et al. (22) have reported that a high-affinity DHEA specific binding site can be found in the cytosol and nuclear fraction in mouse T lymphocytes. The second suggestion is based on the time course of DHEA-induced diacylglycerol production as shown in Fig. 5. DHEA binds to conventional PKC and atypical PKC- ζ such as dexamethasone, as previously reported (17), and activates conventional PKC in the presence of Ca^{2+} and atypical PKC- ζ in vivo and in vitro as shown in Figs. 6–8. Finally, these diacylglycerol-sensitive PKC may possibly act as a phospholipase D activator as previously described (13, 32).

Conversely, DHEA stimulates PI 3-kinase through binding to an unknown tyrosine phosphorylated pro-

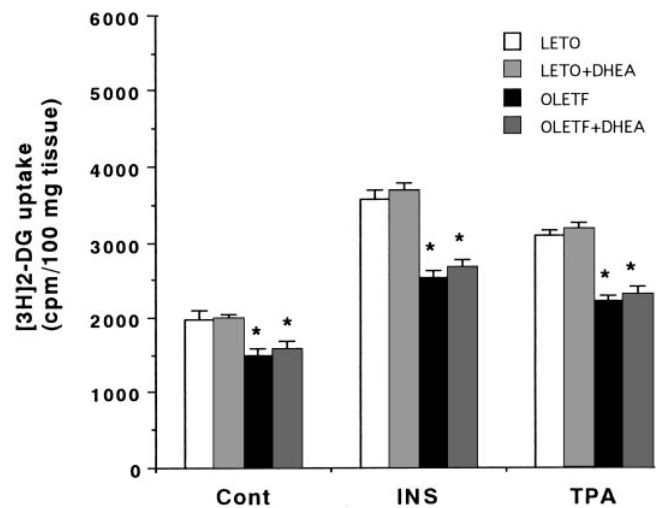


Fig. 10. Effect of in vivo DHEA treatment on insulin- or TPA-stimulated 2- ^3H DG uptake in adipocytes of OLETF/LETO rats at 18 wk of age. Soleus muscles in DHEA-treated and untreated OLETF/LETO rats were incubated in 25 ml Krebs-Ringer bicarbonate buffer for 30 min (Cont) and stimulated with 100 nM insulin (INS) or 1 μM TPA for 30 min; 2- ^3H DG, L-[1- ^{14}C]glucose, and 0.1 mM 2-DG were added, and the incubation was continued for 10 min. After incubation, tissue was removed, rinsed, homogenized in 5% trichloroacetic acid, and counted simultaneously for ^3H and ^{14}C . Data are plotted as means \pm SE of 3 separate experiments. * $P < 0.01$, 2- ^3H DG uptake in OLETF vs. LETO rats by ANOVA with Fisher's PLSD test.

tein unlike insulin as shown in Fig. 4. It should be reasonable to stimulate glucose transport via activation of PKC- ζ by DHEA-induced PI 3-kinase activation. In contrast, dexamethasone does not stimulate glucose uptake, probably due to inhibition of glucose transporter translocation. In fact, a previous report has indicated that dexamethasone suppresses GLUT-4 translocation in rat adipocytes (4). As shown in Fig. 9, in vivo treatment with DHEA results in an increase in insulin-stimulated glucose uptake. The plasma DHEA concentration reaches 10^{-8} to 10^{-7} M in 0.4% DHEA-treated rats as shown in Table 1. This concentration of DHEA significantly enhances insulin- or TPA-stimulated 2-[3 H]DG uptake as indicated in Fig. 2. Therefore, DHEA actually decreases insulin resistance in GK and OLETF rats, NIDDM animal models, without affecting body weight, glucose, or insulin levels. However, we have to consider that there is much less DHEA in rodents than in humans (3). Therefore, it is possible that the effect of in vivo treatment with DHEA may alter insulin resistance dramatically in OLETF rats. On the other hand, it should be noted that insulin- and TPA-induced glucose uptake in soleus muscles of OLETF/LETO rats is not altered by in vivo DHEA treatment for 2 wk. This result may be due to a short period of in vivo DHEA treatment and a weak effect of DHEA on skeletal muscle. Accordingly, it is suggested that DHEA mainly affects adipose tissue as previously reported (10). Further studies will be required on the effect of in vivo treatment with DHEA on diabetic patients.

In conclusion, first, DHEA alone stimulates glucose uptake in rat adipocytes. Second, DHEA stimulates the production of diacylglycerol. Third, pretreatment with DHEA enhances insulin-induced diacylglycerol production. Fourth, DHEA stimulates PI 3-kinase activity, conventional PKC, and atypical PKC. Fifth, specific DHEA binding activity to PKC can be found. Sixth, histone phosphorylation activity increases with the addition of DHEA in the presence and absence of Ca^{2+} . Seventh, treatment with 0.4% DHEA in vivo for 2 wk in GK and OLETF rats results in a significant increase in insulin- and TPA-induced 2-[3 H]DG uptake in adipocytes. Finally, DHEA may be useful in overcoming insulin resistance in NIDDM.

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