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Effect of Dehydroepiandrosterone on Insulin Sensitivity and Adipocyte Growth in Otsuka Long-Evans Tokushima-Fatty Rats

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1. Introduction

Dehydroepiandrosterone (DHEA) (1) is the major adrenal androgen of young adults. However, serum concentration of DHEA in 60 year- old men shows a gradual decrease, when compared with young men aged 25-30 years old. This decrease occurs as the incidence of atherosclerosis (2), obesity (3), and diabetes increases (4), suggesting that administration of DHEA may protect against the development of these disorders. Previously, we reported that in vitro DHEA treatment increased glucose uptake and activation of PI 3-kinase in native rat adipocytes (5).

Protein kinase C (PKC) is a family of serine/threonine kinases, which play key functions in cellular signal transduction. Three categories of PKC, conventional, novel, and atypical PKCs, have been described depending on their mechanisms of activation. Moreover, it is known that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is the hub of many signaling pathways, which phosphorylate many downstream kinases of phosphatidylinositol 3-kinase (PI 3-kinase), such as Akt kinase, S6 kinase and PKC. Previous studies suggested that atypical PKC (aPKCζ/λ) isoforms are required for insulin stimulation of glucose uptake (6), and PDK1 is necessary for activation of aPKCs (7).

It has been reported that DHEA treatment reduces fat accumulation and protects against insulin resistance via an increase in PI 3-kinase after immunoprecipitation with insulin receptor substrate-1 (IRS-1) in male rats (8). We have investigated the in-vitro and in-vivo effects of DHEA on insulin-induced glucose uptake in adipocytes of Otsuka Log-Evans Tokushima fatty (OLETF) (9) and LETO rats. Moreover, we have shown the DHEA-induced glucose uptake by activation of PI 3-kinase/atypaical PKC siganlling without association with IRS-1 (10). In vivo treatment with DHEA affects on a decrease of adipose tissue via downregulation of peroxisome proliferator-activated receptor γ (PPARγ) expression (11). Based on above results, we have searched more precise mechanism of DHEA-induced amelioration of insulin sensitivity and clinical application of DHEA in diabetic animal models and human male adults.

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Obesity is one of the most important public health problems because of its association with increased risk for diabetes, hypertension, coronary heart disease, and other serious diseases. In addition to the environmental factors including a western style diet, hormonal changes are postulated to up- or downregulate adiposity. Recent evidence indicates that hypogonadism is a predictor of obesity, insulin resistance, diabetes, and coronary artery disease in men (12). Testosterone replacement therapy improves insulin resistance and glycemic control in hypogonadal men with type 2 diabetes (13). Male hypogonadism is a distinctly defined clinical entity associated with sexual dysfunction, inactivity, depression, decreased muscle mass, bone loss, etc. Hence, testosterone replacement also improves these abnormalities (14). On the other hand, although circulating DHEA and testosterone concentrations are known to decline during the aging process (15) in men, the relationship between low plasma DHEA level and cardiovascular disease remains controversial. Barrett-Connor and colleagues reported negative correlation between DHEA sulfate (DHEA-S) level and death from any case and death from cardiovascular disease in men more than 50 years of age (16). The same authors concluded that DHEA-S did not predict cardiovascular death in women (17). Analyses of the relationship of plasma DHEA or DHEA-S levels with obesity or body fat distribution have revealed inconsistent results. Plasma DHEA levels are negatively correlated with total body mass index and abdominal fat accumulation; however, contradictory is true for the relationship between DHEA-S and adiposity. Several studies supported the association of high plasma DHEA level with favorable insulin sensitivity (18). Meanwhile, administration of DHEA has been reported to improve obesity (19), diabetes (20), and cancer (21) in animal studies. However, discrepancies have been reported in the benefit of DHEA administration in human studies. Both positive (22) and negative (23) effects of DHEA replacement in elderly people or in patients with adrenal insufficiency have been reported. A major cause of these discrepancies might be the varied study designs including the selection of subjects, dose, drug administration, and definitions of endpoint in these numerous studies.

Although the specific receptor of DHEA has not been identified, one recently proposed mechanism is that DHEA is a precursor of androgen and estrogen that is converted to active form in peripheral tissues. DHEA is metabolized into androstenedione with 3β-hydroxysteroid dehydrogenase, and then transformed to testosterone with 17β-hydroxysteroid dehydrogenase (intracrine process) (24). The fact that androgen receptor (AR)-deficient mice are obese (25) indicates that AR acts as a mediator to prevent fat accumulation in whole body. Accordingly, several effects of DHEA may be mediated via AR. In this regard, it is possible that DHEA and testosterone have common mechanisms to reduce adiposity. However, few comparative studies between these hormones have been reported (26).

We demonstrated that administration of DHEA reduced adiposity in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Moreover, we found that DHEA directly downregulated the PPARγ mRNA level in cultured adipocytes, which may explain the anti-obesity effect of DHEA (11). However, contradictory data have been reported lately (27). To understand the weight-reducing effect of DHEA comprehensively, we performed a microarray study. Analysis of approximately 30,000 genes in epididymal fat isolated from mice after treatment with or without DHEA demonstrated that although a few genes involved in energy metabolism (e.g., malonyl-CoA decarboxylase) were affected, genes regulating cell growth (e.g., foxa1, nuclear factor I/X, eukaryotic translation elongation factor 1α, and aurora kinase) and genes regulating apoptosis (e.g., caspase 3, tumor necrosis factor receptor

superfamily, and Bcl2) were markedly up- and downregulated, respectively. These results indicate that DHEA regulates cell growth or death, possibly resulting in decreased adiposity. However, we failed to find evidence of increased apoptosis in adipose tissue and cultured adipocytes after DHEA treatment.

2. Materials and methods

Materials. [γ-^{32}P]ATP was purchased from Amersham (Aylesbury, Buckinghamshire, UK). [1,2-^{3}H]2-deoxyglucose ([^{3}H]2-DOG) was purchased from DuPont-NEN (Boston, MA). Anti-phosphotyrosine (PY) antibody was purchased from Transduction Laboratory (Lexington, KY). Anti-PKCζ, anti-Akt and anti-PDK1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DHEA was purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

Adipocyte experiments. Males Wistar rats weighing 150-200 g were fed ad libitum and killed by decapitation. Isolated adipocytes were obtained by collagenase digestion of rat epididymal fat pads in Krebs-Ringer phosphate (KRP) buffer (pH 7.4) containing 127 mM NaCl, 12.3 mM NaH$_2$PO$_4$, 5.1 mM KCl, 1.3 mM MgSO$_4$, 1.4 mM CaCl$_2$, 3% bovine serum albumin (BSA), and 2.5 mM glucose (28). Animal care and use were approved by the Committee for Animal Care and Use in Gifu University Graduate School of Medicine.

Transfection study. Plasmids for expression of wt-PKCζ (M246)/ΔPKCζ (K281W mutant) (kindly supplied by Dr. Shigeo Ohno, Yokohama City University School of Medicine, Japan), wt-PDK1/ΔPDK1 (K110N) and wt-Akt/ΔAkt (T308A) (kindly supplied by Dr. Robert V. Farese, University of South Florida College of Medicine, USA) were constructed in vector SRD, pCDNA3 and pCIS, respectively. Adipocytes (50% vol/vol) were transfected by the electroporation method (5 μg DNA/cuvette) as described by Quon et al in the presence of SRD, pCDNA3 or pCIS eukariocytic expression vector clone, or SRD-, pCDNA3- or pCIS-containing cDNA encoding wtPKCζ/ΔPKCζ, wtPDK-1/ΔPDK-1 or wtAkt/ΔAkt (29). Transfected adipocytes were incubated at 37℃ overnight (5% CO2) in Dulbecco's Phosphate-Buffered Saline containing 20 μg/ml ampicilin. After overnight incubation, each protein content was identified by an enhanced chemiluminescence Western blotting detection system according to the manufacturer's protocol. To ensure equal loading of proteins, membrane were stripped and restained with antibodies against β-actin.

PI 3-kinase activity. Isolated rat adipocytes were preincubated with KRP buffer for 30 min. The samples were treated with 1 μM DHEA at 1 min and 10 min, and were lyzed immediately in lysis buffer [20 mM Tris/HCl, pH 7.5, 140 mM NaCl, 20 μM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM Na$_3$VO$_4$, 1 mM ethylene glycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 10% Glycerol, 1% Nonidet P-40, and 10 μg/ml aprotinin]. The lysates were centrifuged at 15,000 $\times$ g for 10 min at 4℃. Five μg of anti-PY antibody was added to 500 μg of the above resultant supernatant and incubated at 4℃ overnight. After addition of protein A sepharose for 2 h, the immunoprecipitates were washed twice with washing buffer A [20 mM Tris/HCl, pH 7.5, 140 mM NaCl, 1 mM dithiothreitol (DTT), and 1% Nonidet P-40], once with LiCl solution (100 mM Tris/HCl, pH 7.5, 0.5 M LiCl, and 1 mM DTT), and twice with buffer B (10 mM Tris/HCl, pH 7.5, 100 mM NaCl, and 1 mM DTT). The PI 3-kinase reaction was started by the addition of a 40 μl solution containing 20 mM Tris/HCl, pH 7.4, 10 μCi [γ-^{32}P]ATP, 250 μM ATP, 10 mM MgCl$_2$, 5 mM EGTA, and 20 μl (1 mg/ml) of sonicated phosphatidylinositol. After a 10 min

incubation at 30℃, the reaction was terminated by the addition of 500 μl 1 N HCl. Then, in succession, 500 μl of $CHCl_3$/methanol (2:1, vol/vol) was added to the samples, which were then centrifuged. The extracts were washed with 500 μl of $CHCl_3$/methanol/1 N HCl (4:2:3, vol/vol), then dried and resuspended in $CHCl_3$. The samples were spotted onto silica gel 60 thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany), and visualized by autoradiography (X-OMAT film, Eastman Kodak, Rochester, NY) (30). These results were then scanned and analyzed with a laser densitometer (UltroScan XL, Pharmacia LKB Biotechnology, Tokyo).

Glucose uptake study Isolated rat adipocytes were incubated with KRP buffer for 30 min, followed by treatment with 1 μM DHEA or 10 nM insulin for 30 min. Uptake of [³H]2-DOG was measured over a 1 min period after treatment with 1 μM DHEA or 10 nM insulin. When overexpressed rat adipocytes were treated with 1 μM DHEA for 30 min, uptake of [³H]2-DOG was measured over a 1 min period after treatment with 1 μM DHEA for 30 min. In another experiment when overexpressed rat adipocytes were incubated with or without 1 μM wortmannin for 10 min, followed by treatment with 1 μM DHEA for 30 min, uptake of [³H]2-DOG was measured over a 1 min period after treatment with 1 μM DHEA for 30 min.

PKC studies Wt-PKCζ-transfected adipocytes were treated with 1 μM DHEA for 10 min, respectively, following pretreatment with or without 1 μM wortmannin, and were terminated by the addition of ice-cold buffer C (20 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 0.1 mM PMSF, 20 μg/ml leupeptin, 20 mM 2-mercaptoethanol, and 10 μg/ml aprotinin). Each sample was sonicated in buffer C. The homogenates were centrifuged for 60 min at 105,000 × g to separate the cytosol and membrane fractions. After the membranes were resuspended in lysis buffer (20 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 20 μg/ml leupeptin, 20 mM 2-mercaptoethanol, 5 mM EGTA, 0.1 mM PMSF, 2 mM ethylenediaminetetraacetic acid, and 1% Triton X-100) for 30 min at 4℃, they were sonicated, and then centrifuged for 60 min at 105,000 × g to obtain solubilized membrane fractions. They were then resuspended in Laemmli sample buffer and boiled for 2 min at 100℃. Thirty μg of each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and subjected to Western analysis with anti-PKCζ antibody as described above. These results were then scanned and analyzed with a laser densitometer.

Animal study

Male Long-Evans Tokushima Otsula (LETO) rats and Otsuka Long-Evans Tokushima Fatty (OLETF) rats were obtained from Otsuka Pharmaceutical Co., Ltd. Animals were fed CE2 powder ad libitum, and treated with (DHEA group) or without (control group) 0.4% DHEA for 52 weeks. They were housed in a specific pathogen-free facility with a 12-h light/12-h dark cycle. All procedures for animal care were carried out in accordance with protocols approved by the University of Gifu's Institutional Animal Care Committee. To compare the effects of DHEA and testosterone on adiposity, male Wistar rats were fed with (control) or without 0.4% DHEA- or 0.4% testosterone-containing food for 4 weeks. Body weight, fat weight, and serum levels of glucose, IRI, DHEA-S, and testosterone were measured.

Statistics

Experimental results were calculated as means ± standard errors. Statistical comparisons were performed by ANOVA. Significance was defined as $P < 0.05$.

3. Results

Overexpression of wild type- and kinase inactive type-PKCζ/PDK1/Akt.

DHEA stimulated both PI 3-kinase activation and DG production as previously described (5). We examined whether DHEA activates PKCζ, which is thought to be a downstream of PI 3-kinase. When isolated adipocytes of LETO rats (control) were treated with or without (control: 0 min) 1 μM DHEA for 5 , 10 and 20 min, cytosolic PKCζ immunoreactivities gradually decreased and membrane-*associated* PKCζ immunoreactivity were inversely increased as previously described (10). Moreover we examined precise mechanism of DHEA action in adipocytes. Although each DNA was transfected to rat adipocytes transiently, these protein contents were significantly overexpressed in rat adipocytes after overnight incubation. Each protein content was identified by an enhanced chemiluminescence Western blotting detection system. Each wild type- and kinase inactive type-PKCζ/PDK1/Akt-transfected cells exhibited a 2 to 3 fold increase in immunoreactive signal of 2-3 fold, compared with nontransfected cells (Fig. 1).

DHEA-induced 2-DOG uptake.

First, we examined DHEA-induced 2-DOG uptake in rat adipocytes. DNA, using the electroporation system was transfected into adipocytes. DHEA-induced 2-DOG uptake in wt-PDK1- and wt-PKCζ-transfected adipocytes significantly increased to 250±30% and 280±50%, respectively, compared with the control cells and vector alone-transfected cells (Fig. 2). However, wt-Akt-transfected adipocytes had no significant influence on DHEA-induced 2-DOG uptake in this system, when compared with the control cells and vector alone-transfected cells (Fig. 2).

Second, we also examined the effect of wortmannin pretreatment on DHEA-induced 2-DOG uptake. DHEA-induced 2-DOG uptake after pretreatment with 1 μM wortmannin for 30 min was significantly reduced in wt-PDK1-transfected adipocytes. Similarly, DHEA-induced 2-DOG uptake after pretreatment with wortmannin for 30 min was significantly reduced in wt-PKCζ-transfected adipocytes (Fig. 2).

DHEA-stimulated PKCζ translocation with or without wortmannin pretreatment.

DHEA-induced membrane-associated PKCζ immunoreactivity for 20 min in PKCζ-transfected cells was greater than that of control cells and tranfected cell without DHEA stimualtion. (Fig. 3, lanes 1 and 2). The DHEA-stimulated PKCζ translocation in PKCζ-transfected cells significantly increased to 3-fold greater than the control (Fig. 3, lane 3). Pretreatment with wortmannin for 15 min completely suppressed DHEA-stimulated PKCζ translocation from the cytosol to the membrane in PKCζ-transfected cells (Fig. 3, lane 4). These results indicate that DHEA-induced PKCζ translocation is provoked via a PI 3-kinase dependent pathway.

Effect of DHEA treatment in vivo on insulin-induced glucose uptake in adipocytes of OLETF/LETO rats

We selected a type 2 diabetes animal model, the OLETF rat, and examined the effect of "in vivo" DHEA treatment on insulin-, TPA- or DHEA-induced [³H]2-DOG uptake. At 10 wk of age, 10 nM insulin-induced [³H]2-DOG uptake in adipocytes of OLETF rat decreased by 45% when compared to LETO rat (control). In the 2 weeks treatment with 0.4% DHEA in vivo, there were no significant differences in body weight or plasma insulin level but there was a
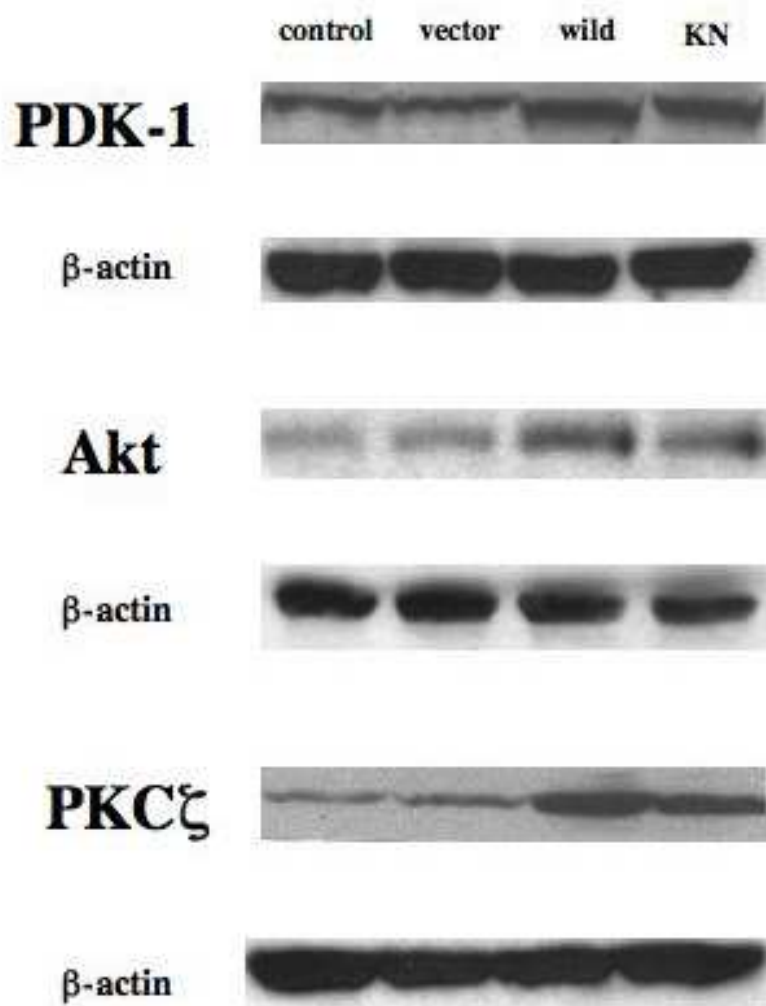
Fig. 1. **Overexpression of PDK1, PKCζ and Akt in rat adipocytes.** Isolated rat adipocytes were prepared as described in "Materials and Methods". Adipocytes (50%) were transfected by the electroporation method (5 µg/cuvette) in the presence or absence (control) of eucariocytes expression vector-containing wild and kinase-inactive (KN) cDNA encoding PDK-1, Akt and PKCζ. Transfected adipocytes were incubated at 37°C overnight in Dulbeco's modified buffer. Each protein was identified by ECL western blotting system as shown in "Materials and Methods". Equal loading of protein was confirmed by stripping the membrane and staining with anti-β-actin antibody. Transfection with wild type and kinase-negative type (KN) PDK1/PKCζ/Akt significantly increased immunoreactive PDK1/PKCζ/Akt 2-3 fold compared with nontransfected cells, respectively. Statistical significance was determined by Fisher's PLSD test.

significant difference in plasma glucose of OLETF rats as shown in Table 1. After "in vivo" treatment with DHEA, insulin-induced glucose uptakes were significantly increased by 50-100% when compared to untreated OLETF and LETO rats, but not in DHEA- induced glucose uptake. TPA-induced glucose uptake was also significantly increased compared to untreated OLETF and LETO rats as previously described (5). These results suggested that DHEA markedly increased glucose transport activity in not only LETO rats, but also in OLETF rats.
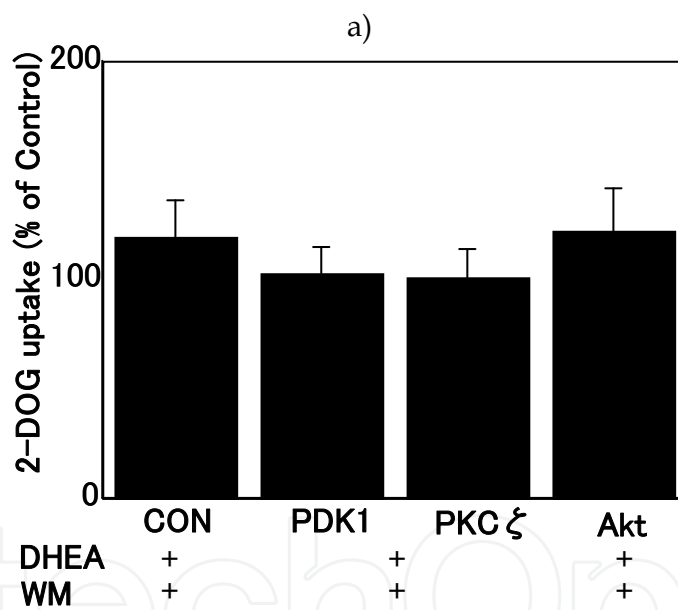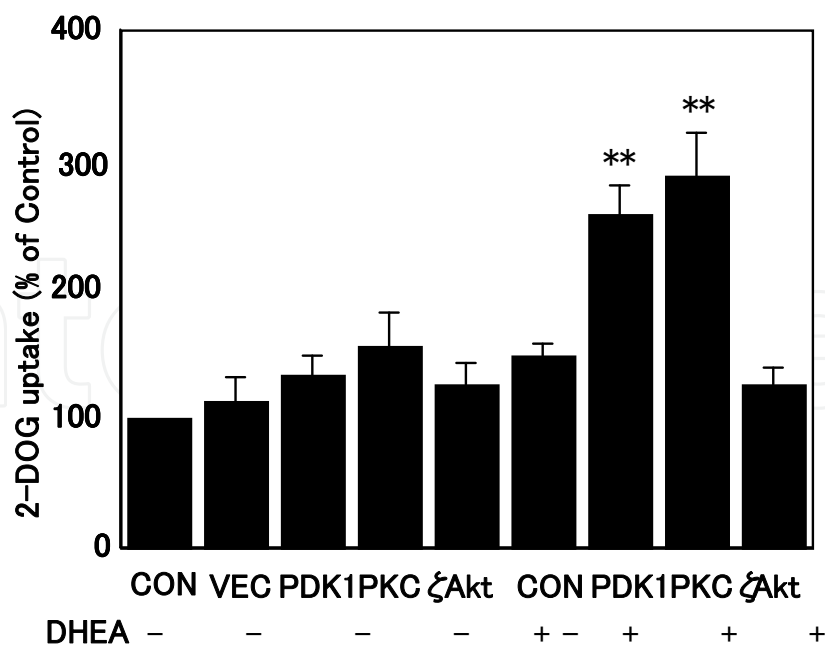
Fig. 2. **DHEA-induced 2-DOG uptake in transfected adipocytes.** a) Isolated adipocytes obtained by the electroporation system were stimulated with 1 μM DHEA for 30 min. Uptake of [³H]2-DOG was measured as shown in "Materials and Methods". DHEA-induced 2-DOG uptake in wt-PDK1- and wt-PKCζ-transfected adipocytes significantly increased to 250±30% and 280±50%, respectively, compared with the control cells and vector alone-transfected cells. b) Isolated adipocytes obtained by above procedure were pretreated with 1 μM wortmannin for 30 min, and then stimulated with 1 μM DHEA. DHEA-induced 2-DOG uptake after pretreatment with wortmannin for 30 min was significantly reduced in wt-PDK1-transfected and wt-PKCζ-transfected adipocytes. The data are plotted as mean ± SE of three separate experiments. **, $P < 0.01$, vs. control by Fisher's PLSD test.
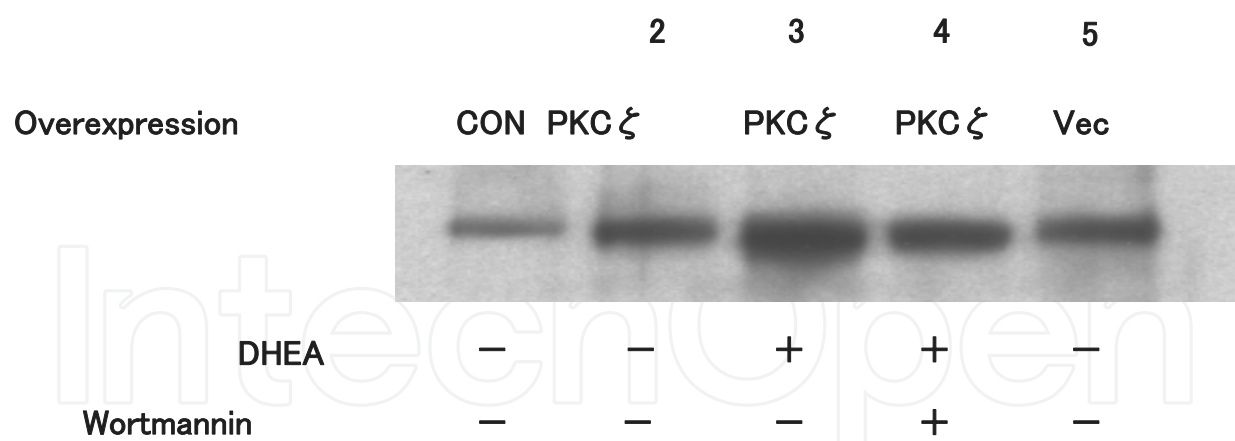
	2	**3**	**4**	**5**
Overexpression	CON PKC ζ	PKC ζ	PKC ζ	Vec

DHEA	−	−	+	+	−
Wortmannin	−	−	−	+	−

Fig. 3. **DHEA-stimulated membrane-associated PKCζ immunoreactivity with or without wortmannin pretreatment.** Adipocytes were transfected wt-PKCζ DNA using an electroporation system. Membrane-associated PKCζ immunoreactivity was examined by Western blotting as shown in "Materials and methods". DHEA-induced membrane-associated PKCζ immunoreactivity in PKCζ-transfected cells significantly increased to about 3-fold over the control (lane 3). Pretreatment with wortmannin for 30 min completely suppressed DHEA-stimulated PKCζ immunoreactivity in PKCζ-transfected cells (lane 4). The result is representative one of three separate experiments.

Specimen	LETO		OLETF	
	Control	**DHEA**	**Control**	**DHEA**
Epididymal fat (g)	10.9 ± 1.4	5.5 ± 1.7**	16.6 ± 1.2	11.2 ± 2.1**
Glucose (mg/mL)	140 ± 1	132 ± 2	184 ± 4	168 ± 3 *
TG (mg/mL)	101 ± 14	53 ± 2 *	544 ± 42	171 ± 54 **
T.Chol (mg/mL)	121 ± 5	139 ± 2	226 ± 7	191 ± 7
FFA (mEq/L)	645 ± 115	490 ± 16	1225 ± 96	854 ± 214 *
AST (IU/L)	207 ± 10	331 ± 35 *	202 ± 49	150 ± 21 *
ALT (IU/L)	75 ± 11	118 ± 9	141 ± 4	101 ± 34 *
IRI (ng/mL)	1.8 ± 0.4	2.4 ± 0.3	2.4 ± 1	2.7 ± 0.4

Data are expressed as mean $\pm$ SE (N = 6).
*$P < 0.05$, **$P < 0.01$ vs control.
Table 1. Effect of DHEA Administration on Epididymal Fat Weight and Laboratory Data in LETO and OLETF Rats

Effect of DHEA treatment in vivo on insulin-induced phosphatidylinositol (PI) 3-kinase and PKCz activations in OLETF/LETO rats

Moreover, in order to clarify the effect of DHEA treatment in vivo on PI 3-kinase, which binds to tyrosine phosphorylated insulin receptor substrate-1 (IRS-1) via SH2 domain of PI 3-kinase, downstream of the insulin signaling pathway, we examined whether DHEA treatment improves PI 3-kinase activity in adipocytes of OLETF/LETO rats. When
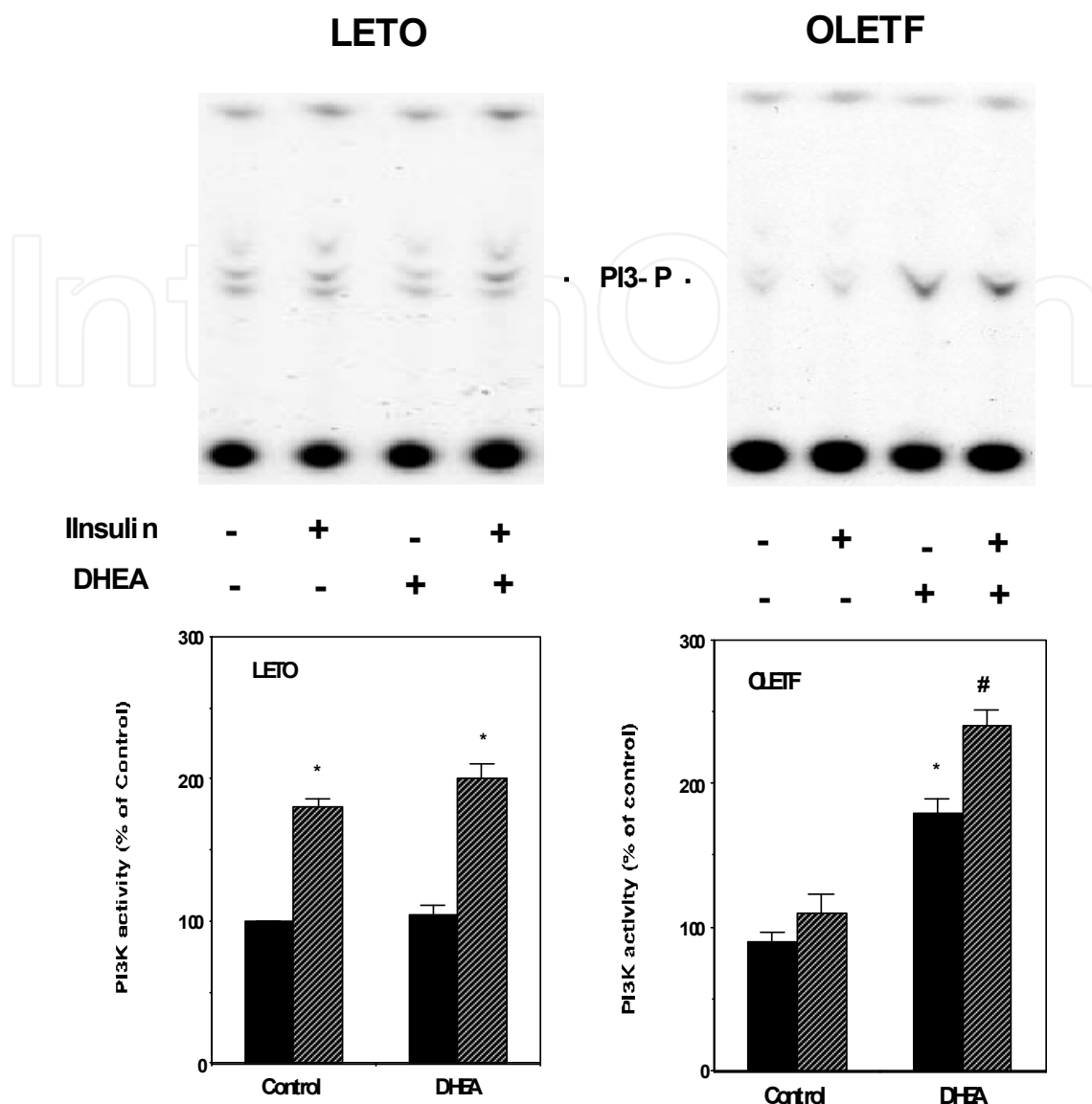
Fig. 4. **Effect of in vivo treatment with DHEA on insulin-induced PI 3-kinase activation in adipocytes of OLETF/LETO rats.** After treatment with 0.4% DHEA in vivo for 2 wk, isolated adipocytes were obtained from epididymal fat pads in OLETF rats. Isolated adipocytes were incubated with or without (control: 0 min) 10 nM insulin for 10 min, homogenized, and immunoprecipitated with phosphotyrosine antibody. Each PI 3-kinase activity was measured as shown in "Materials and methods". Densitometric data are plotted as the mean ± SE of four separate experiments. *P < 0.01 by ANOVA with Fisher's PLSD.

adipocytes were incubated with 10 nM insulin, enzyme activity after immunoprecipitation with antiphosphotyrosine antibody apparently increased for 5 and 10 min in control LETO rats. PI-3kinase activity of OLETF rat was decreased and insulin-stimulated PI-3kinase activity also decreased, compared with those in LETO rats (Fig. 4). In vivo treatment with DHEA increased membrane-associated PKCζ immunoreactivities, especially in OLETF rat as shown in Fig. 5. These results suggest that in vivo treatment with DHEA for 2 weeks increases PI 3-kinase/atypicalPKC signaling and finally ameliorate blood glucose level as shown in Table 1.

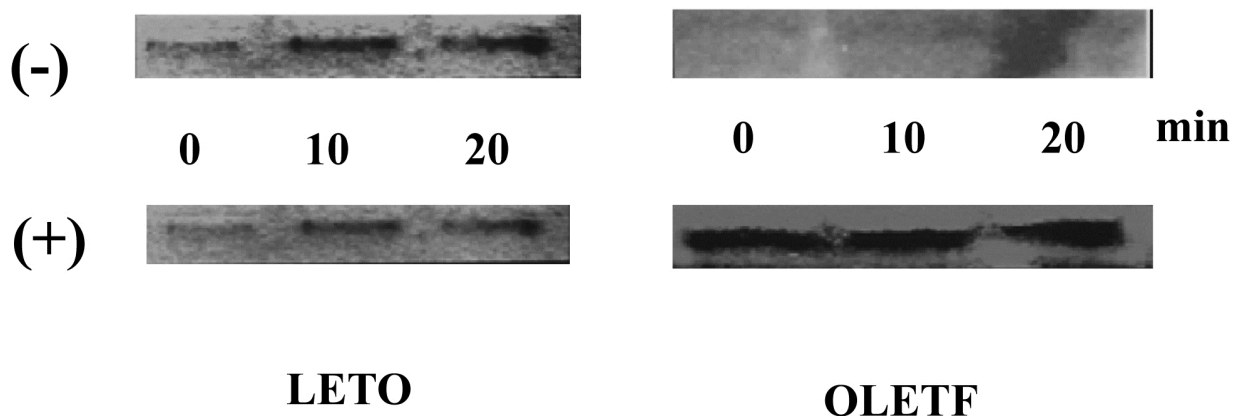# Insulin-induced PKCζ change of membrane fraction in DHEA-treated LETO and OLETF rats

Fig. 5. **Effect of in vivo treatment with DHEA on insulin-induced PKCζ translocations in adipocytes of OLETF/LETO rats.** After treatment with 0.4% DHEA in vivo for 2 wk, isolated adipocytes were obtained from epididymal fat pads in OLETF rats. Isolated adipocytes were stimulated with or without (control) 10 nM insulin for 10, 20 min. Cells were homogenized and centrifuged to obtain cytosol and membrane fraction. Membrane-associated protein (40 μg) was subjected to SDS-PAGE, transferred to the nitrocellulose membrane, and immunoblotted with PKCζ antibodies using ECL system. The result is representative one of three separate experiments.

Effect of DHEA administration on body weight, food consumption, and laboratory Data in LETO and OLETF rats

DHEA administration reduced body weight of both LETO and OLETF rats over a period of 44 weeks; however, a significant decrease in food consumption was observed only in the first 2 weeks. This indicated that DHEA-induced reduction of body weight could not be attributed to the change in food intake (Fig. 6). As shown in Table 1, DHEA administration significantly reduced epididymal fat weight in LETO and OLETF and plasma glucose level in OLETF rats. Serum triglyceride and free fatty acid levels were also diminished by DHEA administration.

Effects of DHEA and testosterone on body weight and fat weight

Treatment with both 0.4% DHEA and 0.4% testosterone resulted in reduction of body weight and epididymal fat, intestinal fat, and perirenal fat weights (Fig. 7A, B). Animals were treated for 4 weeks to avoid the effect of decreased food intake. Because rodents lack 17α-hydroxylase (CYP17A1) in their adrenal glands, they produce little or no DHEA. Hence, DHEA-S was not detected in control serum, whereas elevated DHEA-S levels were observed in both DHEA-treated and testosterone-treated rats (Fig. 7C). Treatment with DHEA resulted in less elevated testosterone levels than treatment with testosterone (Fig. 7D). No differences were observed in serum levels of glucose, triglyceride, free fatty acid, AST, ALT, and IRI levels among these animals (data not shown).
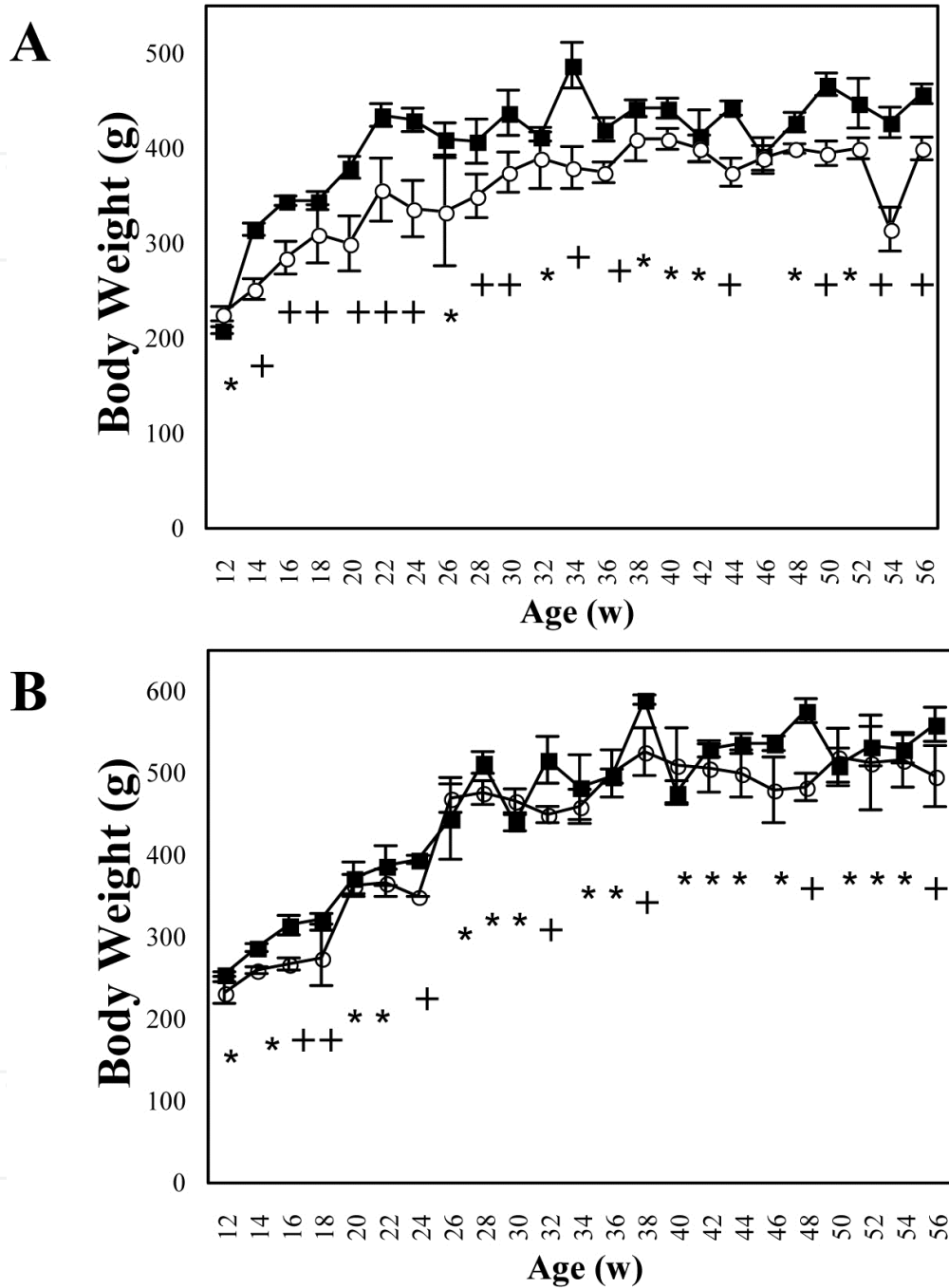
Fig. 6. Effects of DHEA administration on body weight in LETO and OLETF rats
Both LETO and OLETF rats were treated with or without (Control) 0.4% DHEA-containing
food. Effects of this treatment on body weight in LETO (A) and OLETF (B) are shown.
Closed square: Control, Open circle: DHEA, *: $P < 0.05$, vs Control, +: $P < 0.01$, vs Control, N
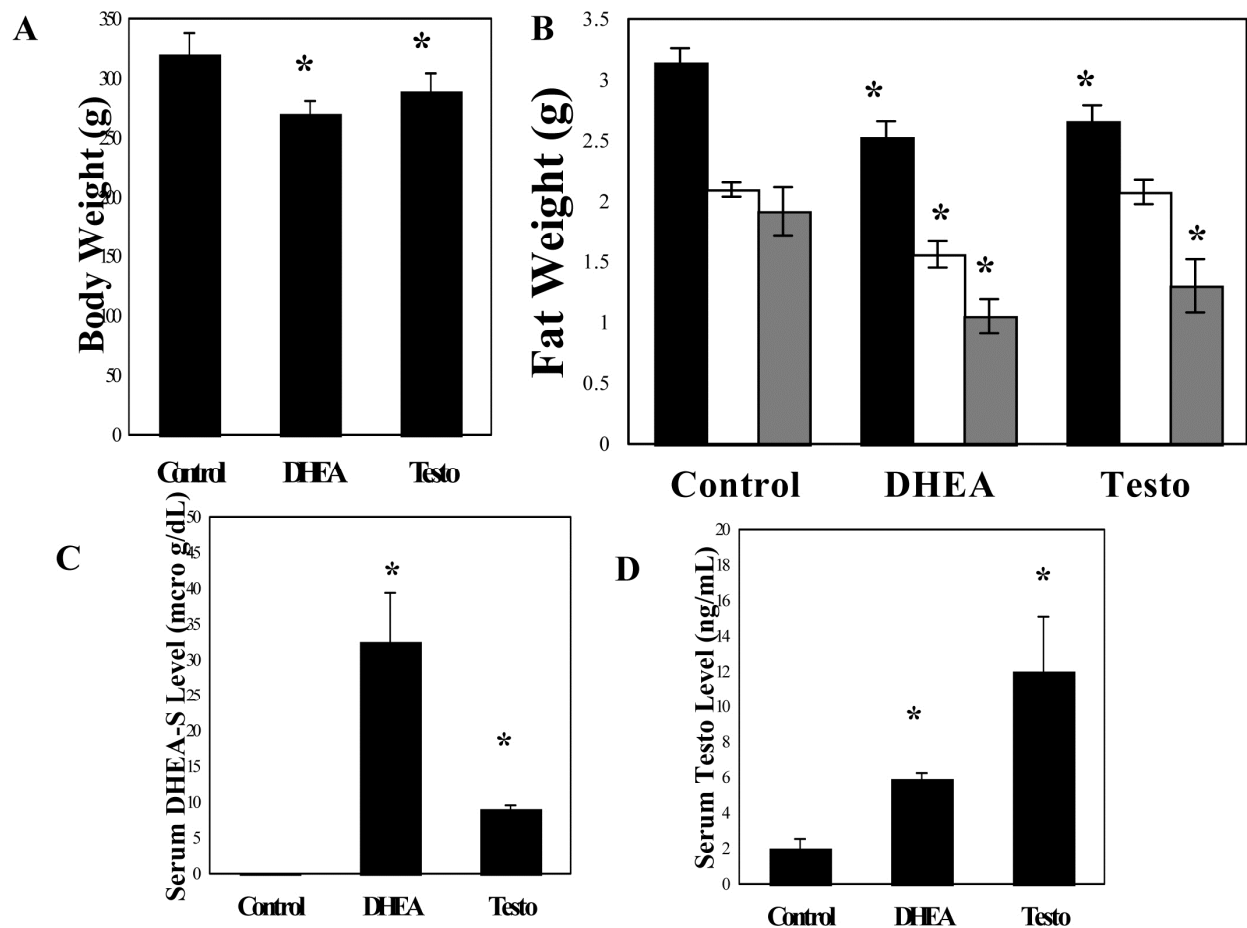= 10.

Fig. 7. Effects of DHEA and testosterone administration on body weight, fat weight, and serum concentration of DHEA-S and testosterone

Wistar rats were housed with or without 0.4% DHEA- or 0.4% testosterone-containing food for 4 weeks. DHEA and testosterone administration produced equal and significant decrease in body weight (A), epididymal (black bar), intestinal (white), and perirenal (gray) fat weight. *: $P < 0.05$ vs each control, N = 8. Testosterone (Testo) administration raised serum DHEA-S level (C), while DHEA administration significantly elevated serum testosterone level (D). *: $P < 0.05$ vs control, N = 6.

4. Discussion

We have previously reported that DHEA improves insulin resistance via activation of PI 3-kinase/atypical PKC signaling (5). However, more precise mechanism still remains unclear concerning about downstream signaling of PI 3-kinase and in vivo effect of DHEA on human male adults. Therefore, we have shown in vivo administration of DHEA stimulates glucose uptake via activation of PI3-kinase/atypiacal in OLETF rats and improves insulin resistance in human male adults. It has been reported that insulin receptor-mediated tyrosine phosphorylation of insulin receptor substrate 1 stimulates PI 3-kinase (31). It is believed that PI 3-kinase plays an important role in the insulin-stimulated glucose transport system (32). It has been reported that PI 3-kinase mediated aPKC activation is essential in insulin-induced glucose transporter 4 translocation and glucose uptake (33). The current results demonstrate that PDK1 is necessary for phosphorylation and activation of aPKC (34),

and aPKC may provoke glucose transporter translocation to the membrane of insulin sensitive tissues (35). Our previous studies show that DHEA may directly or indirectly activate PI 3-kinase without an increase in tyrosine phosphorylation of IRS-1 (36) and subsequent activation of PKCζ (13). Although DOG uptake by DHEA stimulation significantly increased, it appears to be more than the 150% increases in cultured adipocytes from an unstimulated level, as shown in Fig. 2. In this study, we hypothesize that overexpressions of PKCζ and PDK1 may markedly cause DOG uptake. As a result, DHEA-induced DOG uptake increased 250 and 280% in wt-PDK1- and wt-PKCζ-transfected adipocytes, respectively, when compared with the control and vector-transfected cells, as shown in Fig. 2. DHEA-induce glucose uptake was stimulated by activation of PI-3 kinase and subsequent stimulation of PDK1/atypical PKC by PIP_2/PIP_3 production as already shown (5), and partially mimics insulin-induced activation of glucose uptake.

Although we acknowledge that PDK1 can phosphorylate Akt kinase (known as protein kinase B), DHEA-induced DOG uptake did not increase in wt-Akt-transfected adipocytes (Fig. 2). Moreover, Kotani et al stated that Akt kinase was not associated with insulin-induced glucose uptake in 3T3-L1 adipocytes (37). Further work will be needed to focus on the elucidation of the function of Akt kinase.

In addition, we investigated the effect of wortmannin, a PI 3-kinase inhibitor, on glucose uptake in wt-PKCζ-transfected adipocytes. When treated with DHEA, PKCζ significantly increased in the membrane fractions as indicated in Fig. 3. (lane 3). However, pretreatment with wortmannin for 30 min significantly suppressed DHEA-induced PKCζ translocation to the membrane (Fig. 3, lane 4). We also investigated the effect of wortmannin on DHEA-induced PI 3-kinase and PKCζ activity. After pretreatment with wortmannin, DHEA-induced PI 3-kinase and PKCζ activations were inhibited completely as previously described (10). These results indicate that acute DHEA stimulation may potentiate DOG uptake via a PI 3-kinase-PDK1-PKCζ pathway and subsequent glucose transporter translocation.

Nakashima et al. reported that insulin-stimulated DOG uptake increased by incubating cells with DHEA (38). We also revealed that DHEA could enhance insulin-induced DOG uptake via activation of PI 3-kinase-PKCζ signaling in DHEA-treated (for 2 weeks) OLETF (Otsuka Long-Evans Tokushima Fatty) rat in vivo, when compared with untreated control rats (5). Although the precise mechanism is unknown, the preferential activation of PI 3-kinase and redistribution of PKCζ by chronic DHEA treatment may appear to result in enhancement of insulin sensitivity in OLETF rats and human male adults. On the other hand, DHEA converts into estrogenic and androgenic metabolites, such as 17β-estradiol, 5-androstene-3β,17β-diol, testosterone and 4-androstene-3,17-dione (39). Investigators need to examine whether DHEA-metabolites affect DOG uptake, although in vitro examinations indicated androstenedione and estradiol did not stimulate DOG uptake in native rat adipocytes as previously reported (5). Finally, 4-hydroxy-androstenedione, an aromatase inhibitor, may be useful in evaluating the effect of DHEA.

In this study, DHEA treatment for 2 wk in genetically obese OLETF rats reduced the weight of subcutaneous and visceral adipose tissues, but not total body weight. Although a small effect could also be observed also in control rats, DHEA decreased a broad range of adipose in fatty ones. We also revealed that DHEA treatment significantly reduced plasma leptin level as previously reported (11). Leptin is thought to correlate with the amount of stored body. Therefore, DHEA-induced reduction in adipose tissue mass might contribute to the

decrease in plasma leptin concentration. Moreover, we found that DHEA treatment prevented the elevation of PPARγ expression in adipose tissue from OLETF rats (11). The present study could not ascertain whether the expression of PPARγ in adipose tissue is genetically determined or not. However, considering the fact that an increased amount of PPARγ expression in obese human adipose tissue, which is proportional to BMI, can be reduced by a low calorie diet (40), our result seems to suggest an acquired origin. We questioned whether these effects of DHEA on adipose tissue are direct or not. Interestingly, DHEA-S cannot stimulate peroxisomal gene induction in the liver of PPARγ knockout mice, suggesting that some of the DHEA or DHEA-S actions are mediated via the PPAR family (41). These results are supposed to be biologically significant, because Pro 12 Ala substitution in the human PPARγ gene, which exhibits 30-45% less transactivation capacity, leads to prevention of obesity and insulin resistance (24). Our recent preliminary study revealed that the effect of DHEA on PPARγ was enhanced in adipocytes with overexpression of PKCζ (11). We previously reported that DHEA activates phosphatidylinositol 3-kinase (PI 3-kinase) and atypical PKC (10). In addition, TNF-α, another PPARγ reducing agonist, also activated atypical PKC (42).

Based on the above results, DHEA may improve insulin resistance via activation of glucose transport and downregulations of adiposity through the activation of PI 3-kinase/PKCζ signaling and the reduction of PPARγ and, finally, DHEA may contribute to the amelioration of insulin sensitivity in diabetic model animals. These results suggest that DHEA may be useful for clinical application for insulin resistant state such as diabetes, obesity, hyperlipidmia and hypertension, recently called as metabolic syndrome in the future time.

Since the presentation by Coleman and colleagues suggested that DHEA administration prevented diabetes in db/db mice, numerous studies have been published (20). Our previous study revealed that although treatment with DHEA fails to decrease blood glucose in Goto-Kakizaki rats, an animal model of lean type 2 diabetes, it significantly reduces blood glucose in OLETF rats (5). Therefore, we speculated that DHEA administration might improve obesity-associated diabetes in association with preventing obesity-induced insulin resistance. We found that treatment with DHEA for 2 weeks reduced fat mass and that DHEA directly suppressed mRNA levels of PPARγ in isolated adipocytes (11). Since suppression of PPARγ activity leads to reduced adiposity in PPARγ-deficient mice (43) and Pro12Ala polymorphism in humans (44), our result could explain the weight-reducing effect of DHEA. On the other hand, our microarray experiment revealed that genes regulating cell proliferation were more profoundly influenced by DHEA administration than those regulating energy homeostasis in adipose tissue. Hence, we examined the effect of DHEA on cell proliferation in adipose tissue.

In this study, we demonstrated that we compared the effects of DHEA and testosterone administration. Long-term DHEA administration decreased body weight of both LETO and OLETF rats (Fig. 6). Although several studies indicated that the anti-obesity effect of DHEA was associated with decreased food intake, no difference was observed in food consumption except for that in the first 2 weeks. It was suggested that DHEA-induced central neurotransmitter changes suppress the appetite; however, these changes may not persist longer than 3 weeks (45).

Epidemiological studies suggest that high plasma DHEA-S level is associated with longevity (46, 47), whereas, to our knowledge, our study is the first to provide evidence of the anti-aging effect of DHEA. Both DHEA and testosterone led to decreased visceral fat weight

when administered for 4 weeks. These results imply that treatment with these hormones acts similarly on adipose tissue. Moreover, dot blot analysis revealed that both testosterone and DHEA administration yielded identical results. In addition, our results suggest that DHEA administration could substitute for testosterone replacement; however, because DHEA is known to act as a neurosteroid and other unique actions of DHEA are known, further study is necessary to understand the difference between these hormones.

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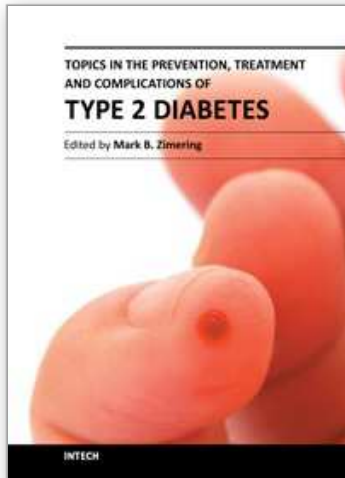
Topics in the Prevention, Treatment and Complications of Type 2 Diabetes

Edited by Prof. Mark Zimering

Type 2 diabetes is estimated to affect 120 million people worldwide- and according to projections from the World Health Organization this number is expected to double over the next two decades. Novel, cost-effective strategies are needed to reverse the global epidemic of obesity which is driving the increased occurrence of type 2 diabetes and to less the burden of diabetic vascular complications. In the current volume, Topics in the Prevention, Treatment and Complications of Type 2 Diabetes, experts in biology and medicine from four different continents contribute important information and cutting-edge scientific knowledge on a variety of topics relevant to the management and prevention of diabetes and related illnesses.

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