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Molecular Differences Caused by Differentiation of 3T3-L1 Preadipocytes in the Presence of either Dehydroepiandrosterone (DHEA) or 7-Oxo-DHEA[†]

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ABSTRACT: The effects of dehydroepiandrosterone (DHEA) and 7-oxo-DHEA on the cell size, adiposity, and fatty acid composition of differentiating 3T3-L1 preadipocyte cells are correlated with stearoyl-CoA desaturase (SCD) expression (mRNA and protein levels) and enzyme activity. Fluorescence-activated cell sorting shows that preadipocyte cells treated with methylisobutylxanthine, dexamethasone, and insulin (MDI) plus DHEA comprise a population distribution of predominantly large cells with reduced adiposity. In contrast, cells treated with MDI plus 7-oxo-DHEA comprise a population distribution of almost equal proportions of small and large cells that have an adiposity equivalent to cells differentiated with MDI alone. The cells treated with MDI plus DHEA have significantly reduced levels of total fatty acid, mainly due to a dramatic reduction in the level of palmitoleic (Δ^9-16:1) acid. The cells treated with MDI plus 7-oxo-DHEA have a significantly increased level of total fat, primarily due to increased levels of Δ^9-16:1 and palmitic (16:0) acids. At the molecular level, the DHEA-treated cells contain lowered amounts of SCD1 mRNA and antibody-detectable desaturase protein, while 7-oxo-DHEA-treated cells contained elevated levels of SCD1 mRNA and protein. Inhibition of differentiation in DHEA-treated cells was also suggested by a reduction in the mRNA level of the adipogenic gene aP2. At the level of microsomal enzymatic activity, SCD activity was decreased in DHEA-treated cells while the SCD activity was increased in 7-oxo-DHEA-treated cells. The changes in mRNA levels and enzyme activity were concentration-dependent and appeared as early as day 3 of the differentiation protocol. The results show that DHEA and 7-oxo-DHEA have distinct modes of action with respect to the complex transcriptional cascade required for differentiation. Furthermore, differences in the insulin-stimulated uptake of 2-deoxyglucose and in the activity of carnitine palmitoyl transferase observed from either DHEA- or 7-oxo-DHEA-treated cells support the ability of DHEA to produce a thermogenic effect in differentiating preadipocytes, while 7-oxo-DHEA promotes differentiation without other changes typical of thermogenesis.

The maintainence of lipid composition is an important aspect of cellular homeostasis. Consequently, many disease states are associated with abnormalities in lipid metabolism, including obesity, cardiovascular disease, non-insulin-dependent diabetes mellitus, hypertension, immune disorders, and others (*1*).

The adipocyte has a central role in lipid metabolism via participation in the storage of triacylglycerols during periods of energy surplus and in the mobilization of fats during periods of deprivation. Adipocytes have been intensively studied in recent years (*2*−*4*), and the mouse embryo-derived 3T3-L1 cell line has often been used as a model system for the study of preadipocyte differentiation, maturation, and cellular processes. With the appropriate hormonal stimulus, 3T3-L1 cells exhibit dramatic increases in gene transcription leading to elevated levels of glycolytic, lipogenic, and lipolytic enzymes and assume many of the morphological properties of mature adipocytes (*5*). Upon differentiation, the total lipid content of fully differentitated 3T3-L1 adipocytes increases by as much as 600%, with the C16 and C18 lipid fraction assuming a recognizable distribution characterized by a predominance of Δ^9-16:1 and lesser amounts of 16:0, Δ^9-18:1, and 18:0 (*6*). SCD,[1] the integral membrane enzyme responsible for introduction of the first double bond into long-chain saturated fatty acids, is an important participant in the differentiation process (*7*). Three isoforms of SCD, encoded by the genes *scd1*, *scd2*, and *scd3* are known (*8*−*10*). These isoforms have 85−88% identity of primary sequence. Two of them, SCD1 and SCD2, are presently known to be present in 3T3-L1 cells. The regulated expression of these isoforms, proposed to have different catalytic selectivites for C16 and C18 acyl chains (*6*, *11*), may contribute to the maintenance and fine control of lipid composition.

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Preadipocyte differentiation arises from a complex transcriptional cascade including the action of C/EBP-β and -γ, PPARγ2 and C/EBP-α, and ADD1/SREBP1 (*3*). The use of synthetic ligands to manipulate expression from this cascade plays a prominent role in current research (*12*) and clinical activities (*13*). The thiazolidinediones represent one such class of synthetic ligands (*14*). TRO, a representative thiazolidinedione, is an agonist for PPARγ2 and strongly promotes the preadipocyte differentiation process (*15*). However, this ligand does not yield a normal lipid composition in 3T3-L1 cells because the lowered level of *scd1* expression (*6*, *16*) apparently yields a reduction in Δ^9-16:1. The identification of additional ligands for the receptors involved in 3T3-L1 differentiation may further elucidate the complexity of this regulatory cascade in addition to suggesting potential theraputic options.

DHEA (Figure 1) and DHEA-sulfate are the most abundant steroids in blood (*17*). They are secreted by the adrenal cortex in response to adrenocorticotropin and serve as precursors to testosterone and estrogens (*17*, *18*). Furthermore, DHEA is converted by liver into a variety of metabolites whose physiological effects remain unclear (*19*−*21*). In recent years, significant interest has arisen from the hypotheses that declining DHEA concentrations in adults may serve as an indicator of a number of conditions including the loss of insulin sensitivity, obesity, diabetes, cardiovascular diseases, stress, and aging, many of which have been correlated with changes in lipid metabolism (*22*). Further characterization of the physiological and molecular bases for these apparent correlations would help to clarify the potential roles of DHEA and its metabolites in cellular metabolism. Figure 1 shows the structure of DHEA and the derivatives examined in this work.

Here we examine the effect of DHEA and the 7-oxo- and hydroxy-DHEA derivatives on the expression, accumulation, and catalytic activity of key lipid-metabolizing enzymes using 3T3-L1 cells as the experimental model. We have proposed that these DHEA derivatives are ergosteroids (*23*) and thus act as thermogenic agents in concordance with many of their observed physiological impacts. P450-catalyzed hydroxylation at the 7-position converts these molecules into a steroid family biosynthetically isolated from the androgens and estrogens (*21*), which may also potentially increase their utility as pharmacological agents (*24*). Our previous studies of 3T3-L1 cells have focused on determining changes in lipid composition, mRNA levels, protein expression, and catalytic activity to broadly characterize the differentiation process (*6*, *7*). This approach has now been used in a study of the molecular effects of DHEA and various 7-position derivatives on 3T3-L1 preadipocyte differentiation. The results show that pharmacological concentrations of DHEA and 7-oxo-DHEA have different consequences on cell size, adiposity, lipogenic gene expression, SCD accumulation, and enzyme activity. These findings indicate that DHEA and 7-oxo-DHEA have distinct modes of action with respect to the transcriptional cascade of differentiation that ultimately result in distinctive lipid compositions. Furthermore, glucose uptake and fatty acid biosynthesis are distinct and consistent with the hypothesis that DHEA provides the most efficient conversion of differentiating preadipocytes to a thermogenic state.

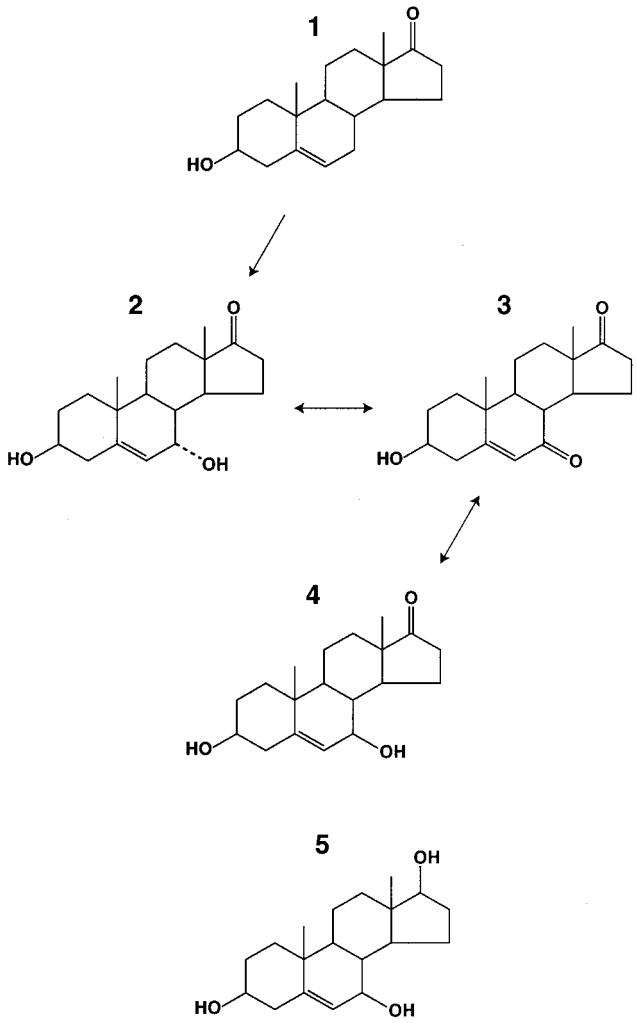

FIGURE 1: Structures of DHEA derivatives investigated: **1**, DHEA, dehydroepiandrosterone or 3β-hydroxyandrost-5-en-17-one; **2**, 7α-hydroxy-DHEA or 3β,7α-dihydroxyandrost-5-en-17-one; **3**, 7-oxo-DHEA or 3β-hydroxyandrost-5-en-7,17-dione; **4**, 7β-hydroxy-DHEA or 3β,7β-dihydroxyandrost-5-en-17-one; **5**, triol-DHEA, 3β,7β,17β-trihydroxyandrost-5-ene.

MATERIALS AND METHODS

Materials. Fetal bovine serum, various preparations of DMEM, TRIZOL reagent, penicillin, streptomycin, and 0.25% trypsin−EDTA were from Gibco BRL (Gaithersburg, MD). Dexamethasone, [1-^{3}H]-2-deoxyglucose (specific activity 5 Ci/mmol), cytochalasin B, sodium deoxycholate, 5,5′-dithiobis(2-nitrobenzoic acid), Tween-20, leupeptin, aprotinin, phenylmethanesulfonyl fluoride, and fatty acid methyl ester standards were from Sigma (St. Louis, MO). Calf serum

[1] Abbreviations: aP2, apolipoprotein isoform 2; C/EBP-α, CCAT/enhancer binding protein alpha; CPT, carnitine palmitoyl transferase; DHEA, dehydroepiandrosterone or 3β-hydroxyandrost-5-en-17-one; 7-oxo-DHEA, 3β-hydroxyandrost-5-en-7,17-dione; 7β-OH-DHEA, 3β,7β-dihydroxyandrost-5-en-17-one; 7α-OH-DHEA, 3β,7α-dihydroxyandrost-5-en-17-one; triol-DHEA, 3β,7β,17β-trihydroxyandrost-5-ene; 16:0, palmitic acid; Δ^9-16:1, palmitoleic acid; 18:0, stearic acid; Δ^9-18:1, oleic acid; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; FAS, fatty acid synthase complex; GF-DMEM, glucose-free DMEM; HG-DMEM, high-glucose DMEM; LG-DMEM, low-glucose DMEM; LXR, liver X-activated receptor; MDI, differentiation cocktail containing methylisobutylxanthine, dexamethasone, and insulin; PBS, phosphate-buffered saline; PPARγ2, peroxisome proliferator-activated receptor γ2; SCD, stearoyl-CoA desaturase; SREBP, sterol response element binding protein; TBS, Tris-buffered saline; TRO, troglitazone.

was from BioWhittaker (Walkersville, MD), insulin was from Eli Lilly (Indianapolis, IN), methylisobutylxanthine was from Aldrich (Milwaukee, WI), Nonidet P-40 was from Calbiochem (La Jolla, CA), and sodium dodecyl sulfate was from Bio-Rad (Hercules, CA). PVDF membranes with a 0.22 μm pore size were from Millipore (Bedford, MA). 9,10-[^{3}H]-Stearoyl-CoA (specific activity 60 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). TRO was a gift from Parke-Davis (Ann Arbor, MI). Protein content of microsomes and mitochondria was determined by dye binding (*25*) using BSA as the standard.

Steroid Derivatives. DHEA was purchased from Steraloids, Inc. (Newport, RI). 7α-OH-DHEA and 7β-OH-DHEA (*26*), 7-oxo-DHEA (*27*), and triol-DHEA (*28*) were synthesized and characterized as previously described.

Culture and Differentiation of 3T3-L1 Cells. The 3T3-L1 preadipocyte cell line was cultured in HG-DMEM with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) in 10 cm Petri dishes until they were 100% confluent. Control cells were cultured in HG-DMEM with 10% fetal bovine serum and antibiotics. For differentiation, 2-day postconfluent cell monolayers (referred to as day 0) were incubated for 48 h in HG-DMEM containing 10% fetal bovine serum, antibiotics, and a differentiation cocktail (MDI) consisting of methylisobutylxanthine (115 μg/mL), dexamethasone (390 ng/mL), and insulin (10 μg/mL). After 48 h, the cells were maintained in HG-DMEM with 10% fetal bovine serum, antibiotics, and insulin only. This medium was changed every 2 days until the cells were collected for analysis. For DHEA and its derivatives, 100 mM stock solutions were prepared in 100% ethanol, filter sterilized with a 0.22 μm pore membrane, and stored at 4 °C. For TRO treatments, a 10 mM stock solution was prepared in dimethyl sulfoxide and added on day 4 to give a final medium concentration of 10 μM. DHEA and its derivatives as well as TRO were replenished with every medium change.

Fluorescence-Activated Cell Sorting. The size and the adiposity of the cells were determined using the fluorescent dye Nile Red (*29*, *30*). Cells were cultured in 6-well plates and differentiated for 10 days. For the FACS experiment, the cells were washed two times with 1 mL of warm PBS and incubated with 100 μL of 0.25% trypsin−EDTA for 1 min at 37 °C. The trypsin solution was then aspirated from the plates, and the cells were incubated for 5 min at 37 °C. The cells were recovered with three 100 μL washes of warm PBS, collected in 5 mL Falcon tubes (Becton Dickinson, Franklin Lakes, NJ), fixed with 100 μL of a 4% paraformaldehyde solution, and immediately put on ice. The cells were stained with Nile Red to a final concentration of 1 μg/mL and kept on ice until the analysis. The samples were analyzed with a FACScan flow cytometer (Beckton-Dickinson, Basel, Switzerland) using 488 nm excitation by an Ar laser. Fluorescence emission was detected at 585 nm. The cell size distribution was determined with a forward scatter (FSC) detector while adiposity (fluorescence of Nile Red) was determined with a side scatter (SSC) detector.

Lipid Analyses. Cells were washed three times with cold PBS, and total cellular lipids were extracted three times with 500 mL of a chloroform/methanol mixture (2:1 v/v). The extractions were combined in a screw-capped glass tube, dried under N_2 gas at 40 °C in a heating block, and resuspended in 50 mL of toluene. Fatty acid methyl esters were obtained by adding BCl_3 in methanol (Alltech, Deerfield, IL) and heating at 80 °C for 30 min. The derivatized fatty acid solution was extracted three times with hexane, dried under N_2, and resuspended in 200 μL of hexane containing 150 μM heptadecanoic acid methyl ester as the internal standard. Fatty acid methyl esters were identified with a Hewlett-Packard (Palo Alto, CA) 6890 gas chromatograph equipped with a 7683 autoinjector and an HP-5 column (30 m × 0.25 mm, 0.25 mm film thickness) connected to a flame ionization detector set at 275 °C. The injector was maintained at 250 °C. The column temperature was held at 180 °C for 2 min after injection, increased to 200 °C at 8 °C/min, held at 200 °C for 15 min, and then increased to 250 °C at 8 °C/min.

mRNA Analyses. Northern blot analyses were performed with total RNA obtained with the TRIZOL reagent (*6*). After agarose−formaldehyde gel electrophoresis and transfer to a nylon membrane, the RNA was hybridized with ^{32}P-labeled cDNA probes synthesized from the divergent 5′ or 3′ untranslated regions specific for either the *scd1* or the *scd2* cDNAs. The membrane was then stripped and sequentially reprobed with the ^{32}P-labeled cDNAs for FAS and aP2. A pAL15 cDNA probe was used as the control for RNA loading.

Western Blot of SCD Protein. The cells were washed with cold PBS, collected, and transferred to microfuge tubes. The cell pellets were lysed by vortexing for 10 s with 500 μL of 50 mM Tris, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM phenylmethanesulfonyl fluoride, 2 μg/mL aprotinin, and 1 μg/mL leupeptin. After mixing, the solution was incubated for 10 min at 4 °C. The cell homogenate was spun at 10000*g* in a tabletop centrifuge at 4 °C for 10 min. The supernatant was transferred to a clean microfuge tube and stored at −80 °C until required for the blotting procedure. The proteins contained in the supernatant (20 μg per lane) were separated by denaturing gel electrophoresis. After the electrophoresis, the proteins were transferred to PVDF membranes in 50 mM Tris, pH 8.3, containing 380 mM glycine, 0.1% SDS, and 20% methanol at 75 V for 45 min. The membrane was washed twice in TBS containing 0.5% Tween-20 and blocked with 5% (w/v) nonfat dry milk in the above-mentioned wash buffer for 45 min at room temperature with constant agitation. The membranes were then washed three times for 3 min.

The primary antibody was a polyclonal antibody raised in rabbits against the last 20 amino acids of the carboxy terminus of rat SCD (*31*). The membrane was incubated with the primary antibody diluted 1:2000 in TBS containing 0.5% Tween-20 and 5% (w/v) nonfat dry milk (blocking buffer) for 1 h at room temperature with constant agitation. The membrane was washed with blocking buffer and incubated with the secondary antibody [goat anti-rabbit IgG−HRP conjugate (Promega, Madison, WI) diluted 1:5000 in blocking buffer] for 45 min at room temperature with constant agitation. The SCD bands were visualized by enhanced chemiluminscence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ).

Preparation of Microsomes and SCD Assay. The cells were washed three times with cold PBS and resuspended in 3 mL of cold 10 mM Tris, pH 7.4, containing 1 mM dithiothreitol and 0.25 M sucrose. The cells were homog-

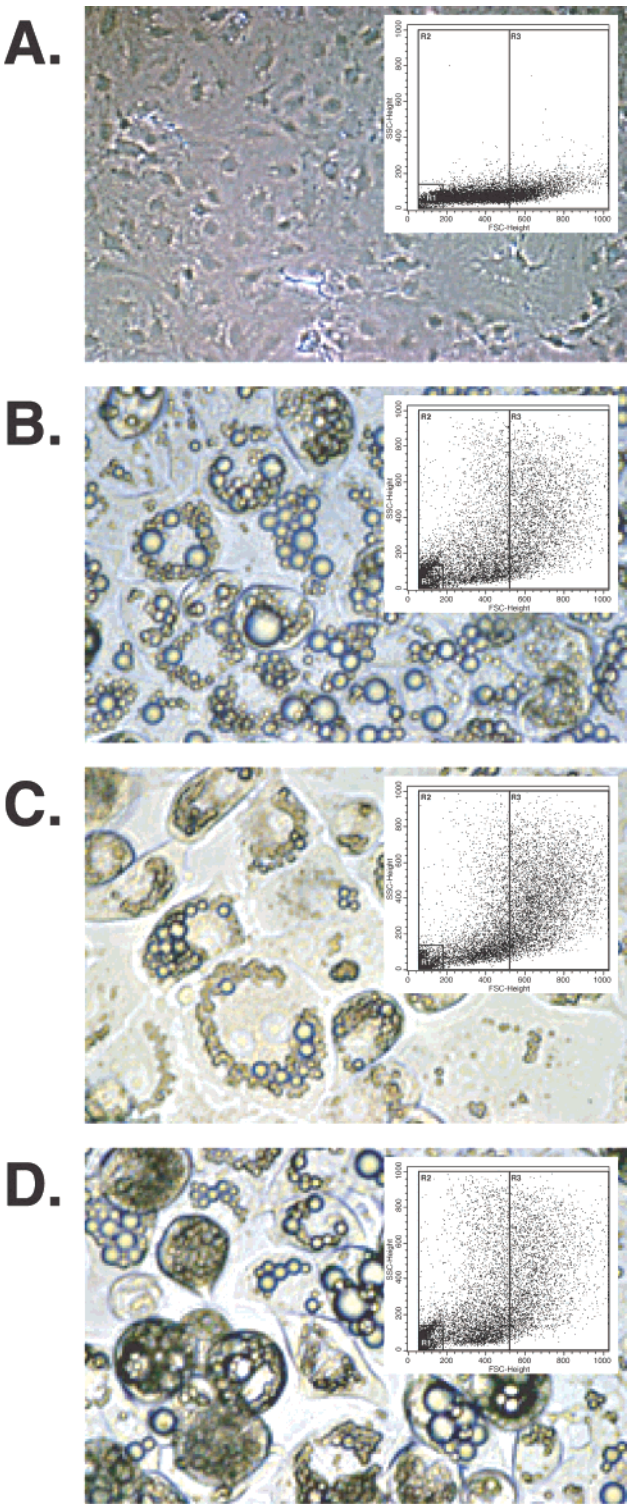

FIGURE 2: Morphology of 3T3-L1 cells revealed by phase contrast microscopy. (A) Day 10 culture of nondifferentiated control cells (magnification 100×). (B) Fully differentiated adipocytes obtained by treatment with MDI (magnification 400×). (C) Adipocytes obtained by treatment with MDI and 100 μM DHEA (magnification 400×). (D) Adipocytes obtained by treatment with MDI and 100 μM 7-oxo-DHEA (magnification 400×). The insets in panels A−D show the results of fluorescence-activated cell sorting.

enized with a variable-speed tissue disruptor (Biospec Products, Inc., Racine, WI) and centrifuged at 15000*g* for 20 min at 4 °C. The cell pellet was discarded, and the supernatant was spun in an ultracentrifuge at 100000*g* for 1 h at 4 °C. The supernatant was discarded, and the microsomal fraction was resuspended in 100 μL of 0.1 M sodium phosphate buffer, pH 7.4. SCD activity was determined from the production of ^3H_2O using [9,10-^{3}H]stearoyl-CoA as the substrate (*32*, *33*) and a Packard TRI-CARB liquid scintillation counter (Model 1600 TR, Downers Grove, IL). To test enzyme inhibition, either 100 μM DHEA or 7-oxo-DHEA was added to microsomes prepared from MDI-treated 3T3-L1 adipocytes harvested at day 10 and assayed for SCD activity as described above.

Carnitine Palmitoyl Transferase Activity. The CPT activity in the mitochondrial fraction was determined as the initial rate of CoA-SH formation upon reaction of palmitoyl-CoA in the presence of (L)-carnitine or (D)-carnitine (*34*). The mitochondrial fraction was obtained essentially as described above for microsomal preparations, except that the mitochondrial pellet was resuspended in 2 mM HEPES, pH 7.4, containing 70 mM sucrose, 220 mM mannitol, and 1 mM EDTA. The assay mixture contained 116 mM Tris-HCl, pH 8, 1 mM NaEDTA, 35 μM palmitoyl-CoA, 0.12 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 1.1 mM of either (L)- or D-carnitine in a total volume of 2 mL. CoA-SH formation was detected using 5,5′-dithiobis(2-nitrobenzoic acid) (*35*). A molar extinction coefficient of 13600 M^{-1} cm^{-1} was used for 5-thio-2-nitrobenzoate. Since CPT is specific for (L)-carnitine, enzyme activity was calculated from the difference between CoA-SH formed in the (L)- and (D)-carnitine reactions. The reaction was initiated by addition of the mitochondrial fraction and monitored at 412 nm for 3 min at room temperature. A linear response was obtained for 2−5 min.

2-Deoxyglucose Uptake Measurements. Cells obtained after 12 days of differentiation were used to investigate insulin-stimulated glucose uptake (*36*). For this experiment, the cells were grown in 6-well plates and differentiated with the MDI differentiation cocktail as described above, with the exception that, from day 6 to day 12, insulin was omitted from the replacement medium. At day 12, the cells were washed once with warm PBS, cultured in serum-free LG-DMEM for 4 h at 37 °C, and then stimulated with either 0, 1, or 100 nM insulin for 30 min at 37 °C in GF-DMEM with 0.5% bovine serum albumin. Glucose uptake was measured using 50 μM [1-^{3}H]-2-deoxyglucose (0.33 mCi/well). After a 10 min incubation, the cells were washed three times with cold PBS, lysed by the addition of 1 mL of 10% Triton X-100, and incubated for 30 min at 37 °C. An 800 μL aliquot of the cell lysate was used for liquid scintillation counting. A correction for noncarrier-mediated uptake was made by measuring the uptake of 50 μM [1-^{3}H]-2-deoxyglucose (0.33 μCi/well) in the presence of 50 μM cytochalasin B, a nonspecific inhibitor of glucose uptake (*37*).

Statistical Analyses. Results were analyzed using the Student's two-tailed *t*-test and the functions provided in Excel 2001 (Microsoft Corp., Redmond, WA). A p value less than 0.05 was considered to be statistically significant.

RESULTS

Morphology of 3T3-L1 Adipocytes. Figure 2A shows nondifferentiated postconfluent 3T3-L1 preadipocytes cells cultured for 10 days without the differentiating cocktail. Cells cultured with DHEA, 7-oxo-DHEA, or any of the three hydroxy-DHEA derivatives alone gave the same morphology. Figure 2B shows the characteristic morphology of mature

Table 1: Fluorescence-Activated Cell Sorting[a] of Differentiating 3T3-L1 Adipocytes Stained with Nile Red

	R2 region (small cells)				R3 region (large cells)			
cell treatment	% of total[b]	cell size[c]	Nile Red fluorescence[d]	normalized adiposity[e]	% of total[b]	cell size[c]	Nile Red fluorescence[d]	normalized adiposity[e]
control[f]	53.7	336	2.8	1	46.3	664	3.2	1
MDI	44.0	334	19.3	6.7	56.0	668	8.3	2.6
+DHEA[g]	35.5	359	**11.4**[h]	**3.9**	**64.5**	675	**4.8**	**1.5**
+7-oxo-DHEA[g]	**51.8**	344	19.4	6.7	48.2	650	9.1	2.8
+TRO[i]	**81.9**	**300**	**10.8**	**3.8**	**18.1**	645	**13.4**	**4.1**

[a] FACS results are the average of $n = 3$ replicate experiments where 10000 cells were counted. The relative standard error for the replicates was less than 5%. [b] Percentage of cells detected in either the R2 or the R3 regions, corresponding to either small or large cells, respectively. [c] Cell size deduced from forward scatter intensity (FSC height). [d] Fluorescence intensity measured as side scatter intensity (SSC height) after staining total cellular lipid with Nile Red. [e] Adiposity indicated by changes in Nile Red fluorescence intensity after normalization to the fluorescence intensity observed from control cells. [f] The control cells were confluent preadipocytes harvested at day 10. [g] DHEA or 7-oxo-DHEA was added to a concentration of 100 μM. [h] Statistically significant differences versus MDI-treated cells within either the R2 or R3 regions are shown in bold ($p < 0.05$, Student's two-tailed *t*-test). [i] TRO (troglitazone, 10 μM) was included as an experimental control since this compound is known to reduce cell size and should thus increase the number of cells detected in the R2 region (*6*, *38*).

adipocytes after culture with MDI for 10 days. These cells were rounded, and the presence of fat droplets was noted. Panels C and D of Figure 2 show the morphology of the adipocytes cultured with MDI plus either DHEA or 7-oxo-DHEA for 10 days, respectively. These cells also showed the characteristic morphology of differentiated adipocytes. However, in contrast to the adipocytes obtained from treatment with MDI alone (Figure 2B), the inclusion of DHEA (Figure 2C) in the growth medium caused a qualitative decrease in the abundance and the size of lipid droplets on the basis of visual inspection. In the case of the cells treated with MDI plus 7-oxo-DHEA (Figure 2D), the cells appeared to be most similar to those obtained by treatment with MDI alone, including the characteristic adipocyte morphology and the presence of abundant fat droplets.

FACS Analysis of 3T3-L1 Adipocytes. Fluorescence-activated cell sorting was used to quantitate the changes in cellular morphology suggested by Figure 2. For each treatment, 10000 cells were cataloged. FSC height measurements indicate the distribution in cell sizes while SSC height measurements indicate the distribution in lipid content. These measurements are plotted in the insets of Figure 2. In the FSC height versus SSC height representation used, the region R1 (FSC and SCC heights less than 200 and 100, respectively) was defined in order to exclude cellular debris from further analysis. Two additional regions of FSC height were defined to accommodate the heterogeneity in observed cell size. These regions are R2 (FSC height of 200−500) and R3 (FSC height of 500−1000). In Table 1, the counting results from the R2 and R3 regions are summarized as the percentage of the total cell count, the arithmetic mean of the FSC height (suggesting the average cell size in the population), and the arithmetic mean of the SSC height (fluorescence intensity of Nile Red, suggesting the adiposity). The SSC height measurements are also normalized relative to the nondifferentiated cell cultures for comparison.

FACS showed that the population of control cells (inset to Figure 2A) exhibited a wide heterogeneity in size and low Nile Red fluorescence, consistent with the nondifferentiated state. Upon differentiation with MDI, and with all other cell treatments investigated, a marked increase in the normalized adiposity was observed, corresponding to the accumulation of lipid upon differentiation. However, the cell size distribution differed depending on the presence of either DHEA, 7-oxo-DHEA, or TRO. Previous studies have reported that treatment of preadipocytes with MDI plus TRO caused smaller cell size and lower total lipid accumulation relative to treatment with MDI alone (*6*, *38*). The statistical analysis of Table 1 quantitates this conclusion. Likewise, treatment of cells with MDI plus 7-oxo-DHEA caused a statistically significant shift in the cell size distribution toward smaller cells but did not cause a decrease in adiposity. In contrast, treatment of cells with MDI plus DHEA caused a statistically significant shift in the cell size distribution toward larger cells but also caused a statistically significant decrease in adiposity.

Differences in Fatty Acid Composition after Differentiation. Table 2 shows the lipid composition of nondifferentiating (control) preadipocytes cells and differentiating adipocytes treated with MDI, MDI plus DHEA, or MDI plus 7-oxo-DHEA.

In nondifferentiating preadipocyte control cultures, ~100 ng of total fatty acids (16:0, Δ^9-16:1, 18:0, and Δ^9-18:1) was detected. The most abundant fatty acid was 18:0 while the least abundant was Δ^9-16:1. The desaturation index, defined as the sum of monounsaturated fatty acids divided by the sum of saturated fatty acids, reflects changes in the lipid composition of the cell as the desaturation activity changes (*39*). For the control culture, the desaturation index was 0.06. Table 2 shows that the addition of DHEA, 7-oxo-DHEA, 7β-OH-DHEA, 7α-OH-DHEA, or triol-DHEA in the absence of MDI produced no statistically significant changes in either the lipid composition or the desaturation index as compared to control cells.

The differentiation of 3T3-L1 cells with MDI for 8 days produced a 6-fold increase in the total lipid content due mainly to an increase in the amount Δ^9-16:1 and Δ^9-18:1. The sum of monounsaturated fatty acids represented almost half of the total fatty acids (48.4%) and a 58-fold increase compared to controls. The increase in unsaturated fatty acid composition corresponded to a higher desaturation index of 1.66. The amount of 16:0 was also higher in the MDI-treated cells, although 16:0 accounted for a similar percentage of the total fatty acid as observed control cultures. The addition of either 7β-OH-DHEA, 7α-OH-DHEA, or triol-DHEA to MDI-treated 3T3-L1 cells produced no significant changes in either the lipid composition or the desaturation index (Table 2).

3T3-L1 cells differentiated with MDI plus DHEA showed a statistically significant decrease in the amount of total fatty

Table 2: Effect of DHEA and Derivatives on the Fatty Acid Composition of Differentiating 3T3-L1 Adipocytes

		control cells plus						MDI-treated cells plus				
fatty acid[a]	control[b]	DHEA[c]	7-oxo-DHEA	7β-OH-DHEA	7α-OH-DHEA	triol-DHEA	MDI	DHEA	7-oxo-DHEA	7β-OH-DHEA	7α-OH-DHEA	triol-DHEA
Δ^9-16:1	1.2 (1.1)	1.4 (1.3)	1.3 (1.2)	1.1 (0.9)	0.9 (0.7)	0.8 (0.9)	231 (36.4)	**100**[d] (20.5)	**270** (38.1)	228 (36.5)	197 (32.9)	198 (30.0)
16:0	26 (24.5)	23 (21.3)	27 (24.5)	27 (24.1)	28 (23.7)	25 (23.3)	139 (21.9)	140 (28.9)	**163** (23.1)	145 (23.2)	149 (24.8)	181 (27.3)
Δ^9-18:1	4.1 (3.9)	5.8 (5.3)	5.5 (5.0)	6.1 (5.4)	7.7 (6.5)	7.9 (7.3)	76 (12.1)	**141** (29.0)	91 (12.8)	82 (13.1)	88 (14.8)	78 (11.8)
18:0	56 (53.0)	52 (48.1)	50 (45.4)	51 (45.1)	56 (48.2)	50 (46.0)	45 (7.1)	**98** (20.1)	52 (7.4)	48 (7.7)	50 (8.3)	53 (8.1)
total fatty acids	106	108	110	113	118	108	634	**486**	**708**	624	598	661
Σ-unsaturated FA	5.3 (5.0)	7.2 (6.6)	6.8 (6.1)	7.2 (6.3)	8.6 (7.2)	8.7 (8.0)	307 (48.4)	241 (49.5)	361 (50.9)	310 (49.6)	285 (47.6)	276 (41.7)
Σ-saturated FA	82 (77.3)	75 (69.4)	77 (70.0)	78 (69.0)	84 (71.1)	75 (69.4)	184 (29.0)	238 (49.9)	215 (30.3)	193 (30.9)	199 (33.2)	234 (35.4)
desaturation index[e]	0.06	0.09	0.08	0.09	0.10	0.11	1.66	1.01	1.67	1.60	1.43	1.18

[a] Individual fatty acids isolated from 8-day cultures reported as nanograms per culture. Numbers in parentheses indicate the percentage contribution to total fatty acids reported. Values shown are the mean of $n \geq 3$ experiments with individual fatty acid analyses repeated in duplicate. In all cases, variances were less than 10%. [b] The control cells were confluent preadipocytes harvested at day 8. [c] DHEA and derivatives were added to a final concentration of 100 μM. [d] Statistically significant differences versus the MDI-treated culture are shown in bold ($p < 0.05$, Student's two-tailed *t*-test). [e] Desaturation index is defined as (∑unsaturated FA)/(∑saturated FA).

Table 3: Concentration Response of DHEA and 7-Oxo-DHEA on the Fatty Acid Composition of Differentiating 3T3-L1 Adipocytes

			MDI-treated cells plus							
			DHEA (μM)				7-oxo-DHEA (μM)			
fatty acid[a]	control[b]	MDI	100	50	10	100 + TRO[c]	100	50	10	100 + TRO
Δ^9-16:1	1.1 (1.2)	166 (33.8)	**84**[d] (19.4)	160 (33.6)	175 (35.1)	**49** (11.7)	**264** (38.6)	**190** (36.3)	154 (32.2)	**198** (35.5)
16:0	22 (25.5)	104 (21.2)	102 (23.6)	100 (20.9)	117 (20.8)	98 (23.4)	**152** (22.3)	110 (21.0)	115 (24.0)	**133** (22.5)
Δ^9-18:1	9 (10.4)	59 (12.1)	**95** (21.9)	73 (15.4)	63 (12.7)	**97** (23.1)	85 (12.4)	63 (12.2)	57 (12.0)	77 (13.1)
18:0	27 (29.1)	32 (6.3)	**53** (12.2)	30 (6.2)	29 (5.8)	**76** (18.1)	45 (6.7)	36 (6.9)	33 (6.9)	37 (6.3)
total FA	94	493	**432**	479	500	**421**	**684**	**524**	480	**594**

[a] Individual fatty acids isolated from day 8 cultures reported as nanograms per culture; Other details as in Table 2. [b] The control cells were confluent preadipocytes harvested at day 8. [c] Troglitazone (TRO, 10 μM) was included as an experimental control since it is known to inhibit adipocyte differentiation as well as decrease SCD expression and the desaturation of 16:0 to Δ^9-16:1 (*6*). [d] Statistically significant differences versus the MDI-treated culture are shown in bold ($p < 0.05$, Student's two-tailed *t*-test).

acids and a decrease in desaturation index to 1.01. These two changes can be mainly attributed to a dramatic reduction in the amount of Δ^9-16:1. In these cells, the 16:0 level remained the same as in the MDI-treated cells, while the Δ^9-18:1 and of 18:0 levels were significantly higher than the MDI-treated cells.

The lipid composition of 3T3-L1 cells differentiated with MDI plus 7-oxo-DHEA was noticeably different from that of cells differentiated with either MDI or MDI plus DHEA (Table 2). In these cells, a statistically significant increase in the level of total fatty acids was observed. This increase was due to markedly higher levels of Δ^9-16:1 and 16:0, while the levels of Δ^9-18:1 and 18:0 were similar to those observed in the MDI-treated cells. Although the MDI plus 7-oxo-DHEA-treated cells had higher amounts of both monounsaturated and saturated fatty acids, the desaturation index of 1.67 was almost identical to that of the MDI-treated cells (Table 2).

A time−course experiment showed that the changes in the fatty acid composition and the percentages of fatty acids described above for DHEA and 7-oxo-DHEA began as early as day 5 of the differentiation protocol (data not shown).

Concentration-Dependent Changes in Lipid Composition. Table 3 shows the fatty acid composition of 3T3-L1 adipocytes after 8 days of culture in the presence of MDI and 10, 50, and 100 μM either DHEA or 7-oxo-DHEA. A DHEA concentration of 100 μM was required to give the characteristic decrease in the amount of total fat and Δ^9-16:1 and increases in the amounts of 18:0 and Δ^9-18:1. For one set of experiments, 10 μM TRO was included in culture medium beginning at day 4 of the differentiation protocol. Previous studies have shown that although TRO increases the differentiation of preadipocytes, it also represses the expression of *scd1*, which leads to a decrease in the desaturation of 16:0 to Δ^9-16:1 (*6*, *16*). The addition of 10 μM TRO enhanced the changes in lipid composition obtained from the DHEA treatment; i.e., even lower levels of total fatty acids and Δ^9-16:1 were observed along with higher levels of 18:0 and Δ^9-18:1.

In cells differentiated with MDI plus 7-oxo-DHEA at concentrations of 50 and 100 μM, but not at 10 μM, significant changes in lipid composition were observed relative to cells treated with MDI alone. Specifically, elevated levels of total fat, 16:0, and Δ^9-16:1 were observed, whereas levels of 18:0 and Δ^9-18:1 were unchanged. The addition of 10 μM TRO to cells treated with 100 μM 7-oxo-DHEA partially reversed the changes in the lipid composition given by MDI plus 7-oxo-DHEA, yielding a decrease in total fat, Δ^9-16:1, and 16:0, while 18:0 and Δ^9-18:1 were relatively unchanged.

Northern Analysis of Differentiating 3T3-L1 Adipocytes. Differentiation of 3T3-L1 preadipocytes is associated with an increase in the level of the SCD1 mRNA, while almost no change in SCD2 mRNA is observed (*6*). Figure 3 shows a northern blot analysis of adipocytes differentiated with MDI alone or with MDI plus either DHEA or 7-oxo-DHEA. cDNA probes for FAS (as an indication of de novo synthesis of fatty acids), aP2 (as an indicator of adipocyte differentiation), and pAL15 (as a control for loading) were also included in the analysis. The adipocytes differentiated with MDI plus

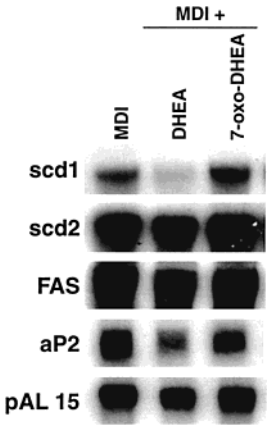

FIGURE 3: Differential effects of either DHEA or 7-oxo-DHEA at 100 μM on mRNA levels detected from *scd1*, *scd2*, a fatty acid synthase gene, and aP2 in MDI-differentiating 3T3-L1 adipocytes. pAL 15 was included as control for loading.

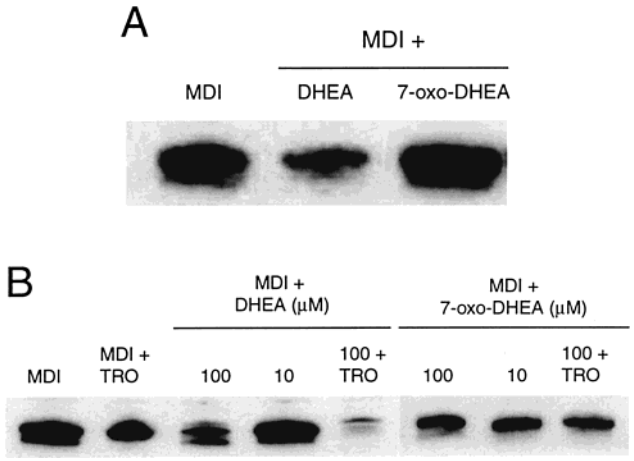

FIGURE 4: Effect of DHEA or 7-oxo-DHEA on SCD protein expression. (A) SCD detected by western blotting in 8-day cultures of MDI-differentiating 3T3-L1 adipocytes where the culture medium was supplemented with either DHEA or 7-oxo-DHEA to give a final concentration of 100 μM. (B) Concentration−response effect of either DHEA or 7-oxo-DHEA on SCD expression as detected by western blotting. TRO (10 μM) was included as an experimental control since it is known to decrease SCD1 mRNA levels, resulting in a decrease in SCD protein levels (*6*, *15*).

DHEA showed substantial decreases in SCD1 and aP2 mRNA levels relative to those detected in MDI-treated cells, while the levels of both the SCD2 and FAS mRNAs did not change. In cells treated with MDI plus 100 μM 7-oxo-DHEA, the mRNA levels for SCD1 were slightly higher, while the mRNA levels of SCD2, FAS, and aP2 were similar to those observed in cells differentiated with MDI alone.

Western Blot Analysis of SCD Expression. Adipocyte differentiation is associated with an increase in the level of antibody-detectable SCD protein (*6*). When added in the absence of MDI, neither DHEA nor any of the DHEA derivatives yielded antibody-detectable SCD protein (data not shown). Figure 4A shows that differentiation of adipocytes with MDI plus DHEA caused an ~60% reduction in the level of antibody-detectable SCD protein, whereas treatment with MDI plus 7-oxo-DHEA produced an ~25% increase in the SCD level when compared to cells treated with MDI alone. The treatment of cells with MDI plus either 7α-OH-DHEA, 7β-OH-DHEA, or triol-DHEA gave a level of antibody-detectable SCD protein similar to that observed in cells treated with MDI alone (data not shown).

The concentration dependence of either DHEA or 7-oxo-DHEA on the antibody-detectable level of SCD protein is shown in Figure 4B. Treatment of cell cultures with MDI plus 100 μM DHEA caused an ~60% decrease in the level of SCD protein, while the addition of 10 μM DHEA had essentially no effect. The addition of 10 μM TRO to cells treated with MDI and 100 μM DHEA resulted in the nearly complete loss of antibody-detectable SCD protein. In contrast to the variation in the level of antibody-detectable SCD protein in cells treated with MDI and DHEA, the amount of SCD protein observed in cells treated with MDI and either 10 or 100 μM 7-oxo-DHEA was similar to that observed in cells treated with MDI alone. Furthermore, this level was only slightly decreased when 10 μM TRO was added to the culture medium.

Effect of DHEA and 7-Oxo-DHEA on SCD and Carnitine Palmitoyl Transferase Activity in Differentiating Adipocytes. Table 4 shows the specific activities measured for SCD and CPT in differentiating adipocytes. Depending on the day of culture, the SCD activity in MDI-treated cells was ~0.6 nmol min^{-1} (mg of protein)$^{-1}$. In these experiments, SCD activity peaked at ca. day 8. The addition of DHEA to the culture medium led to an ~35% reduction in the SCD specific activity. In contrast, the addition of 7-oxo-DHEA to the culture medium produced an ~20−40% increase in the SCD activity. When either DHEA, 7-oxo-DHEA, or ethanol (used as a solvent for DHEA and 7-oxo-DHEA) was exogenously added to the microsomal preparations obtained from day 10 cultures of MDI-treated cells, no change in the SCD specific activity was observed (data not shown).

Since CPT is involved in the translocation of fatty acyl residues from the cytosol into the mitochondrial matrix, this enzyme was used as an indicator of the mitochondrial β-oxidation of fatty acids. The CPT specific activity in cells treated with MDI plus DHEA was ~3-fold higher than that observed in cells treated with MDI alone (Table 4). In contrast, cells treated with MDI plus 7-oxo-DHEA had ~25% lower CPT specific activity than that observed in the cells treated with MDI alone.

Insulin-Stimulated 2-Deoxyglucose Uptake. Table 5 shows the effect that addition of either DHEA, 7-oxo-DHEA, or TRO to the culture medium had on the rate of [1-^{3}H]-2-deoxyglucose uptake. Cytochalasin B, a nonspecific inhibitor of glucose uptake (*37*), was used as a control to correct for nonspecific uptake occurring independent of insulin stimulation. Exposure of control cells to either DHEA or 7-oxo-DHEA alone produced no change in the rate of either basal or insulin-stimulated 2-deoxyglucose uptake relative to control cells (data not shown). When compared to nondifferentiated control cells, the MDI-differentiated cells showed an ~3-fold increase in the basal uptake rate and an ~10-fold increase in the insulin-stimulated uptake rate at both 1 and 100 nM insulin. Cells cultured with MDI plus 100 μM DHEA had a further ~1.5-fold increase in both the basal and insulin-stimulated 2-deoxyglucose uptake rates when compared to the cells treated with MDI alone. In contrast, cells cultured with MDI plus 100 μM 7-oxo-DHEA exhibited slightly reduced rates for the basal and the insulin-stimulated 2-deoxyglucose uptake relative to the cells treated with MDI alone; this decrease was statistically significant at only 1 nM insulin. The addition of 10 μM TRO to cells treated with

Table 4: Effect of DHEA and 7-Oxo-DHEA on the Enzyme Activity of Stearoyl-CoA Desaturase and Carnitine Palmitoyl Transferase in Differentiating 3T3-L1 Adipocytes

	SCD activity[a]			
culture treatment	day 6	day 8	day 10	CPT activity[b]
MDI	0.603 ± 0.01	0.622 ± 0.02	0.591 ± 0.03	1.274 ± 0.01
+DHEA[c]	**0.184 ± 0.01**[d]	**0.227 ± 0.01**	**0.214 ± 0.04**	**3.456 ± 0.16**
+7-oxo-DHEA[c]	**1.042 ± 0.10**	**0.737 ± 0.01**	**0.937 ± 0.03**	**0.807 ± 0.04**

[a] SCD activity expressed as nanomoles of ^3H_2O produced from [9,10-^{3}H]stearoyl-CoA per minute per milligram of protein as described in Materials and Methods. Values shown are the mean ± 1 standard deviation of $n = 3$ determinations. Microsomes were isolated from cultures on the day indicated. [b] Carnitine palmitoyl transferase activity expressed as nanomoles of free CoA-SH produced per minute per milligram of protein. Values shown are the mean ± 1 standard deviation of $n = 3$ determinations. Mitochondrial fractions were obtained from day 10 cultures. [c] DHEA or 7-oxo-DHEA was added to a concentration of 100 μM. [d] Statistically significant differences versus the MDI-treated culture are shown in bold ($p < 0.05$, Student's two-tailed *t*-test).

Table 5: Effect of DHEA and 7-Oxo-DHEA on Insulin-Stimulated 2-Deoxyglucose Uptake in MDI-Differentiating 3T3-L1 Adipocytes

	2-deoxyglucose uptake rate[a]			
		insulin (nM)		
culture treatment	cytochalasin B[b]	0	1	100
control[c]	5.3 ± 0.1	31 ± 4.1	32 ± 3.0	37 ± 3.4
MDI	6.2 ± 0.7	90 ± 4.2	320 ± 34	333 ± 36
+DHEA[d]	**18.2 ± 3.0**[e]	**137 ± 3.9**	**472 ± 13**	**507 ± 42**
+7-oxo-DHEA[d]	8.7 ± 0.6	83 ± 11.3	272 ± 17	307 ± 12
+TRO[f]	9.3 ± 0.5	**131 ± 20.6**	**486 ± 21**	**583 ± 13**

[a] The [1-^{3}H]-2-deoxyglucose uptake rate is reported as picomoles per minute. Results are the mean ± 1 standard deviation of $n = 3$ experiments, with individual experiments performed in triplicate. [b] Cytochalasin B, a nonspecific inhibitor of glucose uptake (*37*), was added to a final concentration of 50 μM. [c] The control cells were confluent preadipocytes harvested at day 12. [d] DHEA or 7-oxo-DHEA was added to a concentration of 100 μM. [e] Statistically significant differences versus MDI are shown in bold ($p < 0.05$, Student's two-tailed *t*-test). [f] Troglitazone (TRO, 10 μM) was included as an experimental control since it is known to increase insulin-stimulated glucose uptake (*15*, *65*).

MDI caused a statistically significant increase in both the basal and the insulin-stimulated uptake rates. In the presence of MDI and TRO, the glucose uptake rates were similar to those obtained with MDI plus DHEA.

DISCUSSION

DHEA and DHEA-sulfate are the most abundant circulating steroids produced by the adrenal cortex, and they are known precursors of the androgenic steroids (*22*). P450 enzymes and other oxidoreductases also convert DHEA into a variety of other metabolites, including 7-hydroxy and 7-oxo derivatives (*21*). The physiological significance of these other DHEA metabolites has not been thoroughly examined. Both the scientific and nonscientific literature have implicated DHEA derivatives in many physiological responses including aging (*40*), cardiovascular disease (*41*), energy metabolism (*23*, *42*, *43*), immunological function (*44*, *45*), memory (*46*), nervous system function (*47*, *48*), obesity (*49*), stress (*50*), and others. Here we have investigated the effect of DHEA, the 7-position hydroxy and oxo isomers, and a triol-DHEA on preadipocyte differentiation using 3T3-L1 cells as the experimental model. In agreement with previous studies (*51*), the present work shows that none of the DHEA derivatives tested was able to initiate the differentiation of 3T3-L1 preadipocytes in the absence of MDI. Moreover, among the derivatives tested, only DHEA and 7-oxo-DHEA gave statistically significant changes in the cellular and molecular properties that we have used to characterize the differentiation of 3T3-L1 cells. These changes occurred in the ~100 μM concentration regime. In normal adult humans, DHEA and DHEA-sulfate have a typical circulating concentration of 3−10 μM (*22*), which is 90% lower than the concentrations used to elicit effects from the mouse 3T3-L1 model system studied here. However, administration of various DHEA derivatives to humans has given up to 20-fold increases in the circulating levels of DHEA and DHEA-sulfate (*22*), which would more closely match the concentrations of the analogues used in this work. Since the Dietary Supplement Health and Education Act of 1994 permits self-administration of these compounds, which are readily available in a nonprescription format, it is possible that concentrations of total DHEA and DHEA-sulfate approaching 100 μM may be observed in humans.

Role of DHEA Derivatives in Preadipocyte Differentiation. DHEA-sulfate does not decrease preadipocyte differentiation, presumably because of the absence of an effective transporter system (*52*). In contrast, high concentrations of DHEA can reduce the proliferation and differentiation of 3T3-L1 preadipocytes (*43*, *52*−*55*). DHEA is known to act as an uncompetitive inhibitor of glucose-6-phosphate dehydrogenase (*42*), which would potentially limit the availability of NADPH required for the de novo synthesis of fatty acids and other intermediates of the pentose phosphate pathway, particularly 6-phosphogluconate. However, no decrease in the NADPH/$NADP^+$ ratio was observed in tissues of rats treated with DHEA (*56*). DHEA-treated 3T3-L1 cells have decreased glucose-6-phosphate dehydrogenase activity, and the addition of exogenous 6-phosphogluconate partially eliminated the DHEA-dependent block of cell growth and differentiation (*43*). An examination of the DHEA precursor pregnenolone, DHEA, and androgenic steroids suggested that DHEA and/or 17β-estradiol (or subsequent metabolites) were (was) likely candidate(s) responsible for the observed decreases in proliferation and differentiation, glucose-6-phosphate dehydrogenase activity, and total lipid content (*52*).

The present FACS analysis (Table 1) show that cells treated with MDI plus TRO gave predominantly small cells with reduced adiposity. These measurements correlate with a long-standing qualitative assessment of the changes in cell morphology and adiposity obtained by cotreatment with MDI and thiazolidinediones (*6*, *38*) and also provide a useful frame of reference for considering the FACS results obtained with the DHEA derivatives used here. Treatment of preadipocytes with MDI or MDI plus 7-oxo-DHEA gave a population of almost equal proportions of small and large cells with similar adiposity for the two treatments. In contrast, the treatment with MDI plus DHEA gave a population of predominantly large cells with reduced adiposity. Furthermore, the adiposity of the MDI plus DHEA-treated cells was significantly lower than either the MDI-treated or MDI plus 7-oxo-DHEA-treated cells.

mRNA Levels. Our previous studies showed that TRO specifically reduced the level of *scd1* mRNA but did not change the level of *scd2* mRNA (*6*). This work shows that DHEA and 7-oxo-DHEA can also modulate the expression of lipogenic genes targeted for differentiation by treatment with MDI (Figure 3). The addition of DHEA caused a dramatic reduction in the detected levels of the SCD1 mRNA and the aP2 mRNA, while essentially no change was observed for the SCD2 and FAS-specific mRNAs. These results are similar to those observed with addition of TRO. In contrast, the addition of 7-oxo-DHEA gave slightly increased mRNA levels for SCD1 and SCD2 and equivalent levels for the aP2- and FAS-specific mRNAs. Thus DHEA and 7-oxo-DHEA both influence the transcriptional cascade leading to differentiation, but with essentially opposite outcomes.

SCD Protein Levels and Catalytic Activity. SCD protein levels and catalytic activity were significantly different in the microsomal fractions prepared from cells differentiated with MDI versus MDI plus DHEA or MDI plus 7-oxo-DHEA (Figure 4). The trends in SCD protein levels and desaturase catalytic activity correlated with the *scd1* mRNA levels observed in the northern blot studies (Figure 3). Thus microsomes prepared from cells treated with MDI plus DHEA had only approximately one-third the SCD specific activity found in microsomes prepared from cells treated with MDI alone. In contrast, microsomes prepared from cells treated with MDI plus 7-oxo-DHEA had ~1.3-fold higher SCD specific activity than microsomes prepared from cells treated with MDI alone. With respect to fatty acid desaturation, these results show that the effects of DHEA and 7-oxo-DHEA on mRNA levels extend to protein translation and the accumulation of enzyme activity.

Lipid Composition. Table 2 shows that the treatment of differentiating 3T3-L1 cells with MDI and either DHEA or 7-oxo-DHEA led to markedly different lipid compositions in the resultant cells. With respect to the results obtained from differentiation with MDI alone, the combination of MDI plus DHEA caused a statistically significant decrease in lipid accumulation (Tables 1 and 2), with the amount of Δ^9-16:1 most dramatically changed. The decreased level of Δ^9-16:1 observed in the MDI plus DHEA-treated cells corresponded with an increase in the levels of 18:0 and Δ^9-18:1. These changes apparently arise from elongation of an excess 16:0 to 18:0 and further desaturation of 18:0 to 18:1. This altered lipid composition is most similar to that obtained by differentiation with MDI plus TRO. Alternatively, the combination of MDI plus 7-oxo-DHEA gave a statistically significant increase in total lipid accumulation, with the resultant lipid composition essentially indistinguishable from that obtained by treatment with MDI alone. In this case, elevated *scd1* mRNA levels (and SCD protein) lead to an accumulation of the long-chain fatty acid pool as Δ^9-16:1.

DHEA Metabolites and Thermogenesis. Considerable evidence supports the conclusion that DHEA and 7-oxo-DHEA act as thermogenic agents in whole animals and in the liver (*23*, *57*). The present studies are also consistent with the action of DHEA as a thermogenic agent in adipocytes (Tables 4 and 5 and ref *58*). Specifically, glucose uptake (both basal and insulin stimulated) and CPT activity were increased by 30−50%, while total fat accumulation was decreased by ~30% (Table 2). Along with the similarities in altered lipid composition described above, cells treated with MDI plus TRO also exhibited increased glucose uptake (*15*) and CPT activity (Tables 4 and 5). These results indicate that DHEA and TRO exert similar thermogenic effects in differentiating adipocytes.

Surprisingly, 7-oxo-DHEA behaves in a different manner by stimulating differentiation, increasing the transcription of lipogenic genes encoding aP2, fatty acid synthase, *scd1*, and *scd2*, giving increased fatty acid biosynthesis, and a higher specific activity for SCD. These events lead to enhanced fat deposition, which would ostensibly be an opposite outcome to that expected for a thermogenic agent. Moreover, glucose uptake and CPT activity are both decreased in 3T3-L1 adipocytes treated with MDI plus 7-oxo-DHEA, suggesting further alterations in glycolysis and fatty acid β-oxidation. With respect to 3T3-L1 cells, 7-oxo-DHEA thus appears to act as a nonthermogenic enhancer of differentiation.

Synergy between DHEA Derivatives and Troglitazones. Table 3 suggests that DHEA and TRO act synergistically to lower lipid accumulation in 3T3-L1 cells. In principle, the action of DHEA to uncouple the glycolytic pathway from fatty acid biosynthesis (*23*) and the action of TRO to alter differentiation mediated by PPARγ2 (*3*) should lead to decreases in lipid formation and alterations in composition. However, the changes in *scd1* and aP2 mRNA levels (Figure 3) indicate that DHEA can also influence the function of the adipogenesis transcriptional cascade. The mouse *scd1* gene contains response elements to SREBP1, C/EBP-α and coactivators such as SP1, NF-Y, and NF-1 but not LXR (*59*). However, the LXR response elements can indirectly affect scd1 transcription via interactions with SREBP-1c (*60*). It is therefore notable that the structural analogue 7-oxo-DHEA appears to have an opposite effect on adipocyte differentiation and thermogenesis. These disparate effects suggest that DHEA and 7-oxo-DHEA may be targeting different aspects of the differentation program. The targets of these ligands are presently unknown, but the participation of ADD1/SREBP1 (*3*) and oxysterol receptor LXRα (*61*, *62*), steroidogenic enzymes (*63*), or the interaction of DHEA derivatives with glucocorticoid-activated transcription factors (*64*) represents attractive options for future investigation.

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