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Mechanism of inhibition of growth of 3T3-L1 fibroblasts and their differentiation to adipocytes by dehydroepiandrosterone and related steroids: Role of glucose-6-phosphate dehydrogenase

(pentose phosphate pathway/6-phosphogluconate/liposomes/epiandrosterone/16α-bromoepiandrosterone)

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ABSTRACT **Dehydroepiandrosterone (DHEA) and certain structural analogues block the differentiation of 3T3-L1 mouse embryo fibroblasts to adipocytes. These steroids also are potent uncompetitive inhibitors of mammalian glucose-6-phosphate dehydrogenases (G6PDs). We provide direct evidence that treatment of the 3T3-L1 cells with DHEA and its analogues results in intracellular inhibition of G6PD, which is associated with the block of differentiation: (*i*) Levels of 6-phosphogluconate and other products of the pentose phosphate pathway are decreased; (*ii*) the magnitude of these decreases depends on the potency of steroids as inhibitors of G6PD and on concentration and duration of exposure, and it is accompanied by a proportionate block of differentiation; (*iii*) in cells exposed to 16α-bromoepiandrosterone (a more potent inhibitor of G6PD than DHEA) at concentrations that block differentiation, introduction of exogenous 6-phosphogluconate in liposomes raises the levels of 6-phosphogluconate and other products of the pentose phosphate pathway and partially relieves the steroid block of cell growth and differentiation.**

Dehydroepiandrosterone (DHEA) is a weakly androgenic and weakly estrogenic steroid that is present in human plasma and urine and is especially abundant as its sulfate (DHEAS) (1, 2). Concentrations of DHEA and DHEAS decline continuously with age (3, 4), and low levels of DHEA and/or DHEAS have been associated with the presence or risk of developing cancer and with increased mortality from cardiovascular disease (5–7). Hence, it is an intriguing possibility that DHEA may exercise hitherto unrecognized regulatory functions, and that some diseases associated with aging may result from a relative deficiency of DHEA or related steroids. These speculations are bolstered by animal experiments. In rodents, administration of DHEA decreases the incidence of spontaneous and carcinogen-induced tumors, retards atherosclerosis, slows weight gain without affecting food intake, ameliorates autoimmune disease, and increases life span (8–10). The mechanisms by which DHEA exerts these actions are unclear. An unusual property of DHEA and several closely related steroids is their potent uncompetitive inhibition of mammalian glucose-6-phosphate dehydrogenases (G6PDs) (11–13). Uncompetitive inhibition, in which the inhibitor binds only to the enzyme–substrate complex, is extremely rare. Inhibition results in proportionate decreases in K_m and V_{max} values and may have profound effects on the flux and levels of intermediates in a multienzyme pathway (13). The possible relationship between inhibition of G6PD and the mechanism of action of DHEA is the subject of this report.

The unequivocal demonstration that inhibition of an enzyme is responsible for a biological effect observed *in vivo* is often difficult. We have used cultures of the 3T3-L1 clone of mouse embryo fibroblasts to obviate some of the complexities encountered with intact animals. Confluent 3T3-L1 monolayers rapidly differentiate into adipocytes when exposed to an appropriate differentiation mixture, and glycerol-3-phosphate dehydrogenase levels provide a simple measure of the extent of differentiation (14, 15). DHEA and related steroidal inhibitors of G6PD block this differentiation process (16, 17). We provide strong evidence in this paper that depression of pentose phosphate levels by steroids contributes to block of differentiation. Thus, intracellular levels of 6-phosphogluconate (6PG) and other intermediates of the pentose phosphate pathway are depressed by steroid treatment, and introduction of 6PG by means of liposomes into such cells restores these levels and reverses the block of differentiation.

MATERIALS AND METHODS

Materials. All reagents were from Sigma. Lipids were from Avanti Polar Lipids. The sources of all other materials have been described (16).

Standard Protocol for Differentiation of 3T3-L1 Cells. Cultures were grown as monolayers on plastic dishes (usually 10-cm diameter) in Dulbecco's modified Eagle's medium containing 10% calf serum (15). Cells were plated at a density of 1.4×10^3 cells per cm^2 and maintained at 37°C in a humidified atmosphere of 10% CO_2/90% air. The medium was replaced on days 3, 5, 7, 9, and 11. To induce differentiation, the medium on day 7 contained 10% fetal calf serum, insulin (10 μg/ml), dexamethasone (1.0 μM), and 1-methyl-3-isobutylxanthine (0.5 mM), and on days 9 and 11 the medium contained 10% fetal calf serum and insulin (10 μg/ml). Additions of blocking steroids [in ethanol or dimethyl sulfoxide; 0.1% (vol/vol)] were made simultaneously with the differentiation mixture on day 7 (16, 17).

The cells were always harvested on day 12. The cell layers were washed three times with cold phosphate-buffered saline (PBS; 150 mM NaCl/10 mM sodium/potassium phosphate, pH 7.4) and then incubated at 25°C for 5 min with small volumes of 100 mM triethanolamine hydrochloride (pH 7.4) containing digitonin (0.8 mg/ml). The supernatant fluids obtained after centrifugation at 20,000 × *g* for 40 min at 4°C were assayed for glycerol-3-phosphate dehydrogenase (16) and for protein concentration (18).

Preparation of Liposomes. Unilamellar anionic liposomes were prepared either by vaporization of petroleum ether solutions (19) or by high-pressure filtration through a 2-μm

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Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; 6PG, 6-phosphogluconate; DHEA, dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one); DHEAS, dehydroepiandrosterone-3-sulfate; EA, epiandrosterone (3β-hydroxy-5α-androstan-17-one); BrEA, 16α-bromo-3β-hydroxy-5α-androstan-17-one.

polycarbonate filter with the use of an "extruder" (Lipex Biomembranes, Vancouver, BC, Canada) (20) of lipid mixtures containing phosphatidylcholine, dicetylphosphate, and cholesterol (molar ratio, 7:2:1). The aqueous phase to be trapped was either PBS or 10–50 mM 6PG in PBS. Liposomes were sonicated for 5 min at 4°C with the microtip of a Branson sonifier at a setting of 5 and 70% power output. Liposomes were concentrated and the untrapped 6PG was removed by filtration through a C-30 Centricon cartridge (Amicon; 30,000 MW cutoff) at 5000 × *g*. The concentration of 6PG trapped in the liposomes was determined enzymatically (21) in the presence of 0.1% Triton X-100. In a typical experiment, 5% of the 6PG contained initially in the aqueous medium was entrapped by the liposomes.

Measurement of Phosphorylated Intermediates. Perchloric acid extracts of cell monolayers (22) were treated with activated charcoal and filtered through Millipore HV 0.45-μm filters to reduce the background fluorescence. Phosphorylated intermediates were assayed enzymatically (21, 23).

RESULTS

Evidence for the Intracellular Inhibition of G6PD by DHEA and Related Steroids. The ability of DHEA and its structural analogues to block differentiation of 3T3-L1 fibroblasts to adipocytes is correlated with the potency of these steroids as inhibitors of G6PDs (16, 17). To provide more conclusive evidence that inhibition of G6PD underlies the block of differentiation, four steroids with increasing potency of inhibition of G6PD were studied: DHEAS, DHEA, and epiandrosterone (EA), which occur naturally, and 16α-bromoepiandrosterone (BrEA), a synthetic steroid. All four steroids inhibited partially purified G6PD from 3T3-L1 cells in an uncompetitive manner with respect to both glucose 6-phosphate and NADP, and with K_i values that were comparable to those for the G6PD of rat mammary gland (11) (Table 1).

Inhibition of G6PD *in vivo* should deplete 6PG levels, since this product of the G6PD reaction is not known to arise by any other metabolic route. When 3T3-L1 cells were treated with DHEA, EA, or BrEA on day 7 simultaneously with the differentiation mixture, the intracellular 6PG levels were indeed depressed by at least 65% during the first 8 hr when the critical events involved in the programming for differentiation to adipocytes occur (17) (Table 2). If exposure to the differentiation mixture and steroid was continued for the standard 48 hr, differentiation also was inhibited, with glycerol-3-phosphate dehydrogenase activities at least 35% lower than those of fully differentiated controls on day 12. In contrast, DHEAS, the least potent inhibitor of G6PD, had no effect on either 6PG levels or on the differentiation process (Table 2). Based on these findings, we selected BrEA, the most potent inhibitor of G6PD examined, as the model compound for the following mechanistic studies.

Table 1. Inhibitor constants (K_i) of DHEA and structural analogues for partially purified G6PDs from 3T3-L1 mouse embryo fibroblasts and lactating rat mammary gland

	K_i for G6PD of		
	3T3-L1 cells		Mammary
Steroid	G6P,* μM	NADP,* μM	gland (11), μM
DHEA	15.0	20.4	18.5
EA	5.79	5.63	3.56
BrEA	0.43	0.62	0.57
DHEAS	1280	—	310

The G6PD was partially purified from differentiated 3T3-L1 cells by ammonium sulfate precipitation and gel filtration. Fluorometric assay mixtures contained 100 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 100 μM NADP (or 0.5–4.0 μM when varied), 1.5 mM G6P (or 10–40 μM when varied), and enzyme (5.1 μg of protein; 1.6 milliunits). Steroids were added in dimethyl sulfoxide (0.1%). The Wilkinson hyperbolic method (24) gave K_m values of 31.1 μM for G6P and 3.4 μM for NADP. The inhibitions with each steroid were strictly uncompetitive with respect to both NADP and G6P. K_i values were determined according to Segel (25).
*Variable substrate.

Table 2. Effects of DHEA and structural analogues on intracellular levels of 6PG in 3T3-L1 mouse embryo fibroblasts and on their differentiation to adipocytes

Steroid	Concentration, μM	Intracellular 6PG, pmol per 10^6 cells	Extent of differentiation, %
None	—	157 ± 5 (100%)	100
DHEA	250	47.6 ± 2.5 (30.3%)	65.0
EA	250	38.1 ± 3.6 (24.3%)	1.0
BrEA	45	57.8 ± 16.6 (36.8%)	19.4
DHEAS	250	172 ± 11 (109%)	108

On day 7, two identical sets of 3T3-L1 cells on 10-cm plates were exposed simultaneously to the differentiation mixture and to the steroids at the concentrations indicated. After 8 hr, the 6PG content of one set of cells was measured. Exposure of the second set of plates to the differentiation mixture and steroids was continued for the standard 48 hr, and the cytosolic glycerol-3-phosphate dehydrogenase specific activity was determined on day 12 and is expressed as percentage of fully differentiated controls. Each measurement is the mean of three plates (±SEM).

Effects of BrEA on Pentose Phosphate Pathway Intermediates. Treatment of 3T3-L1 cells with 30 or 45 μM BrEA for 6–12 hr on day 7 simultaneously with the differentiation mixture resulted in a concentration- and time-dependent depletion of 6PG levels and a proportionate block of ultimate differentiation to adipocytes (Fig. 1). We have shown that exposure to BrEA for 8–12 hr on day 7 is nearly as effective in blocking differentiation as exposure for 48 hr (days 7–9) (17). A drop of nearly 70% in 6PG levels was observed after exposure to 45 μM BrEA for 12 hr, which blocked differen-

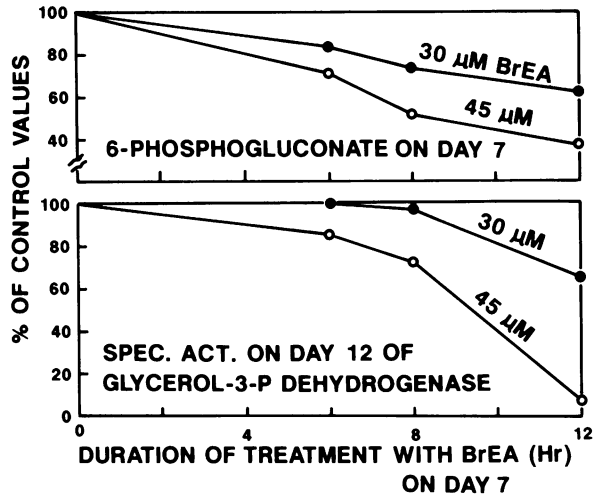

FIG. 1. Effect of BrEA on 6PG levels and differentiation of 3T3-L1 mouse embryo fibroblasts. On day 7, confluent monolayers were treated with differentiation mixture and 30 or 45 μM BrEA for 6–12 hr. (*Upper*) Levels of intracellular 6PG expressed as a percentage of control cells treated with differentiation mixture only (at 6 hr, 200 ± 43 pmol per 10^6 cells; at 8 hr, 285 ± 51 pmol per 10^6 cells; at 12 hr, 335 ± 45 pmol per 10^6 cells). (*Lower*) Extent of differentiation of cells treated with steroid for 6–12 hr and with differentiation mixture for the standard 48 hr. The extent of differentiation was measured on day 12 and is expressed as percentage of fully differentiated control. The mean control value of glycerol-3-phosphate dehydrogenase of differentiating cells was 229 nmol of NADH oxidized/(min·mg of protein).

tiation almost completely. In contrast, the glucose 6-phosphate levels after 12 hr of steroid treatment (and at 6 and 8 hr; data not shown) were not significantly different from those of differentiating controls that had not been treated with blocking steroids (Table 3). Presumably, any accumulation of glucose 6-phosphate resulting from inhibition of G6PD was balanced by diversion of this sugar phosphate into other metabolic pathways such as glycogen synthesis and glycolysis.

The levels of other downstream products of the pentose phosphate pathway were also depressed by steroid treatment (Table 3). Thus, exposure of differentiating 3T3-L1 cells to 45 μM BrEA for 12 hr also lowered the levels of ribulose 5-phosphate and xylulose 5-phosphate to 35–38% of the differentiating controls, and the combined levels of ribose 5-phosphate and sedoheptulose 7-phosphate were lowered to 57% of such controls. These results suggest that although the sugar phosphates of the pentose phosphate pathway are equilibrated with the hexose phosphates of glycolysis by transaldolase and transketolase, these reactions are unable to maintain normal levels of the 5-carbon sugar pool in the presence of a steroid that inhibits G6PD and blocks differentiation.

Protection by 6PG Against Steroid-Induced Block of Differentiation. If depression of 6PG levels and other intermediates of the pentose phosphate pathway plays a fundamental role in the block of differentiation of 3T3-L1 cells by steroids, then elevating these levels by introducing exogenous 6PG into the cells should restore differentiation. Monolayers were exposed to the differentiation mixture for 48 hr (days 7–9) in the presence of 40 μM BrEA alone or together with liposomes containing 10 mM 6PG in PBS (Table 4). The glycerol-3-phosphate dehydrogenase activity on day 12 in steroid-treated cells was only 9.1% of fully differentiated control cells, compared with 48.9% of control values when liposomes containing 6PG were also present (Table 4). Addition of high concentrations of 6PG, of liposomes containing only PBS, or of mixtures of PBS liposomes with high concentrations of free 6PG, had little effect on the steroid block of differentiation. Liposomes containing PBS or 6PG did not affect differentiation in the absence of steroid and were not toxic to undifferentiated control cells.

Protection by 6PG Against Cytotoxic Concentrations of BrEA. Liposomes containing only PBS protected slightly against the block of differentiation by steroids (Table 4), probably because of their ability to sequester steroids, thereby in effect lowering the steroid concentration in the medium (data not shown). To minimize this complication, we studied the effects of much higher (cytotoxic) concentrations of BrEA. Addition of 60 μM BrEA to 3T3-L1 cells for 48 hr (days 7–9) together with the differentiation mixture resulted in almost total cell death. However, if liposomes containing 6PG also were added, the cells were substantially protected against the steroid toxicity, and the surviving cells differentiated partially (Fig. 2). The degree of protection observed depended on the concentration of 6PG-containing liposomes. Thus, in the presence of 60 μM BrEA and liposomes containing 6PG equivalent to 80 μM in the medium, the specific activity of glycerol-3-phosphate dehydrogenase on day 12 was 43% of the activity of fully differentiated control cells. Even at concentrations as low as 20 μM 6PG in the medium, the glycerol-3-phosphate dehydrogenase specific activity was 3-fold greater than that of undifferentiated fibroblasts. If PBS liposomes containing an equivalent amount of lipid were

Table 3. Sugar phosphate levels in 3T3-L1 mouse embryo fibroblasts stimulated to differentiate to adipocytes and exposed to BrEA

Sugar phosphate	Sugar phosphate content of cells, pmol per 10^6 cells		
		Treated with BrEA	
	Untreated	30 μM	45 μM
Glucose 6-phosphate	1770 ± 42	1690 ± 97	1850 ± 83
6PG	335 ± 44	206 ± 35	127 ± 23
Xylulose 5-phosphate	79 ± 9	51 ± 3	29 ± 6
Ribulose 5-phosphate	118 ± 11	56 ± 4	41 ± 3
Ribose 5-phosphate and sedoheptulose 7-phosphate	169 ± 17	131 ± 16	97 ± 21

On day 7, 3T3-L1 cells were exposed for 12 hr to the differentiation mixture alone, or with 30 or 45 μM BrEA, and assayed for sugar phosphates. Cell counts on parallel 10-cm plates were 4.44 ± 0.2 × 10^6 for differentiating controls and 4.23 ± 0.4 × 10^6 and 4.17 ± 0.2 × 10^6 for cultures treated with 30 and 45 μM BrEA, respectively. Each value is the mean (±SEM) of four or five experiments.

Table 4. Protection of 3T3-L1 mouse embryo fibroblasts by liposomes containing 6PG against block of differentiation by BrEA

Addition(s)	Number of measurements	Extent of differentiation, %
None	11	9.1 ± 2.8
Free 6PG (10 mM)	7	3.3 ± 3.0
Liposomes containing PBS	6	17.2 ± 6.5
Free 6PG (10 mM) and liposomes containing PBS	6	14.1 ± 6.4
Liposomes containing 6PG (10 mM)	7	48.9 ± 9.1

3T3-L1 cells in 15-mm diameter wells were exposed for 48 hr (days 7–9) to the differentiation mixture with or without 40 μM BrEA. Cells in parallel steroid-treated wells were also exposed to free 6PG (10 mM), liposomes containing PBS, free 6PG (10 mM) and liposomes containing PBS, or liposomes with entrapped 6PG (final concentration of 6PG in medium, 90 μM). The liposomes were made from a petroleum ether solution containing 8.7 mg of total lipid per ml. Extent of differentiation was measured on day 12 as the specific activity of cytosolic glycerol-3-phosphate dehydrogenase, expressed as a percentage of fully differentiated controls [124 ± 22 nmol of NADH oxidized/(min·mg of protein)].

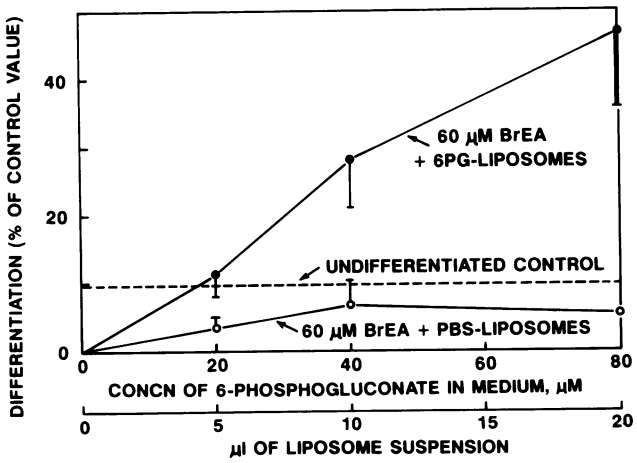

FIG. 2. Protection of 3T3-L1 mouse embryo fibroblasts against cytotoxicity of BrEA by addition of 6PG-containing liposomes simultaneously with the differentiation mixture. The liposomes were prepared by the extruder technique from lipid mixtures (50 mg/ml) and an aqueous phase containing either 50 mM 6PG in PBS or PBS alone. The volumes of liposomes added were 5–20 μl per 500 μl of medium. The final concentrations of added 6PG in the medium were 20–80 μM. The liposomes, steroid, and differentiation mixture were present for 48 hr (days 7–9). The extent of differentiation on day 12 was determined from the specific activity of glycerol-3-phosphate dehydrogenase and is expressed as a percentage of differentiated controls [153 ± 40 nmol of NADH oxidized/(min·mg of protein)]. Exposure to both steroid and differentiation mixture in the absence of liposomes was cytotoxic and thus no measurements were made.

used, the specific activity of glycerol-3-phosphate dehydrogenase was equal to or lower than that of undifferentiated cells.

Transport and Metabolism of 6PG Introduced into 3T3-L1 Cells by Means of Liposomes. Although addition of liposomes containing 6PG protects against the block of differentiation of 3T3-L1 cells by BrEA, evidence is required that this addition raises the levels of intracellular 6PG and subsequent products of the pentose phosphate pathway. Hence, we measured the uptake of 6PG into the cells from liposomes and its conversion to other intermediates. Monolayers of 3T3-L1 cells were exposed on day 7 to the differentiation mixture: alone, with 60 μM BrEA, or with 60 μM BrEA and liposomes containing 6PG to give a final concentration equivalent to 65 μM 6PG in the medium. After 8 hr, the cells were washed twice with medium containing 10% fetal calf serum (equilibrated at 37°C) to remove unincorporated liposomes, and intracellular intermediates of the pentose phosphate pathway were measured. Control cells receiving differentiation mixture alone contained 347 ± 27 pmol of 6PG per 10^6 cells (Fig. 3), whereas with 60 μM BrEA the concentration of 6PG was depressed to 99.5 ± 10.8 pmol per 10^6 cells. When 6PG-containing liposomes were added as well, the intracellular concentration of 6PG was raised to 958 ± 80 pmol per 10^6 cells—i.e., 10 times that of cells treated with steroid and differentiation mixture only. Exposure of cells under the same conditions to either 10 mM 6PG alone or to 10 mM 6PG and liposomes containing PBS had no effect on intracellular 6PG levels (data not shown). In parallel plates exposed to the same combination of 60 μM BrEA and 6PG-containing liposomes for 48 hr, the blocking effect of the steroid was partially overcome, and the glycerol-3-phosphate dehydrogenase specific activity on day 12 was 32% of fully differentiated cells.

If the products of the pentose phosphate pathway are essential for the differentiation of 3T3-L1 cells, then the 6PG taken up into the cells should be metabolized and thereby restore the intracellular concentrations of other downstream intermediates. We measured xylulose 5-phosphate, ribulose 5-phosphate, and the sum of ribose 5-phosphate and sedoheptulose 7-phosphate in liposome-treated and control cells under the same conditions as those described above (Fig. 3). After 8 hr, steroid treatment depressed the intracellular levels of each sugar in comparison to differentiating cells. In contrast, if cells were treated with steroid in the presence of liposomes containing 6PG, intracellular levels of all sugar phosphates were similar to or higher than those of differentiating cells.

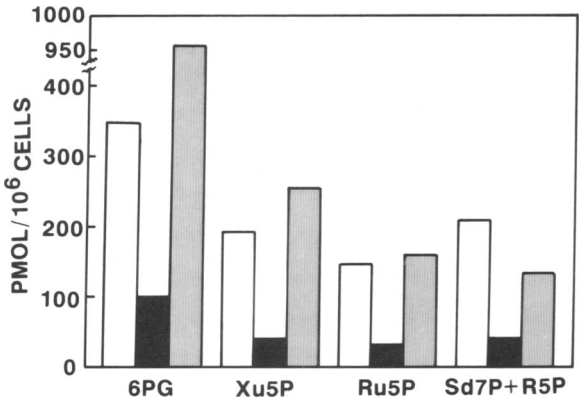

FIG. 3. Depression by 60 μM BrEA of intracellular levels of 6PG, xylulose 5-phosphate (Xu5P), ribulose 5-phosphate (Ru5P), and the sum of ribose 5-phosphate (R5P) and sedoheptulose 7-phosphate (Sd7P) in differentiating 3T3-L1 mouse embryo fibroblasts, and restoration of levels by treatment with liposomes containing 6PG. The liposomes were prepared by the ether evaporation technique with 50 mg of lipid mixture per ml and 50 mM 6PG in PBS as the aqueous phase. The sugar phosphates were measured on perchloric acid extracts after 8 hr on day 7: □, untreated; ■, treated with 60 μM BrEA; ▨, treated with 60 μM BrEA and liposomes containing 6PG (final concentration in medium, 65 μM 6PG). Similar results (not shown) were obtained at 0.5 and 2 hr.

Since liposomes containing 6PG reversed the effects of BrEA and raised intracellular 6PG levels, these experiments strongly suggest that adequate 6PG levels are essential for normal differentiation and that 6PG depletion by inhibition of G6PD can account for the blocking of differentiation by steroids.

DISCUSSION

The inhibition of G6PD by DHEA and related steroids cannot easily explain the protective actions of DHEA against genetically determined diabetes and obesity, restraint of autoimmune phenomena, memory improvement, or immunodulation (for reviews, see refs. 8 and 17). However, at least some of the effects of DHEA *in vivo* and *in vitro* appear to be mediated through inhibition of the pentose phosphate pathway. The two crucial consequences of this inhibition are restrictions on the availability of NADPH (for reductive synthesis and xenobiotic metabolism) and of 5-carbon sugars (for synthesis of ribonucleosides and deoxyribonucleosides) (26). Since supply of exogenous mixtures of deoxyribonucleosides and of ribonucleosides can overcome some of the effects of DHEA and related steroids on differentiation (9, 17), on normal growth (27), and on neoplasia (28), the major effect of DHEA in these systems is apparently to restrict the supply of pentose phosphates. In other systems blocked by DHEA, such as metabolic activation of carcinogens and binding to DNA, as well as superoxide radical generation, it seems more likely that the major result of inhibition of G6PD by steroids is to limit the supply of NADPH. For example, Schwartz and Perantoni (29) have shown that DHEA protects cultured cells against carcinogens such as 7,12-dimethylbenz-[*a*]anthracene and aflatoxin B_1 by inhibiting their activation by NADPH-dependent cytochromes P-450. Similarly, phorbol esters may enhance tumor promotion by stimulating production of superoxide radicals by NADPH-dependent oxidases, and DHEA blocks superoxide generation in phorbol ester-stimulated granulocytes (30).

If decreased G6PD activity is protective against carcinogens, cells from individuals with the severe G6PD deficiency would be expected to behave similarly to normal cells that have been treated with DHEA. Cells from individuals with the severe Mediterranean variant of G6PD deficiency show depression of metabolism, DNA-binding and cytotoxicity of benzo[*a*]pyrene. Furthermore, G6PD-deficient granulocytes generated lower amounts of superoxide in response to phorbol esters (31, 32). All of these effects may result from a limitation of intracellular NADPH supply stemming from a reduction in G6PD activity. Moreover, resistance to benzo[*a*]pyrene cytotoxicity can be mimicked in normal fibroblasts by treatment with DHEA (31–34). Epidemiologic evidence also supports this hypothesis since individuals with G6PD deficiency may be more resistant to developing cancer than normal individuals from the same geographical area (for reviews, see refs. 8 and 9).

In conclusion, there is now considerable evidence that many of the effects of DHEA and its analogues on growth and differentiation, and on the activation of carcinogens, can be attributed to inhibition of the G6PD reaction.

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