Inhibition of Protein Isoprenylation and p21\textsuperscript{ras} Membrane Association by Dehydroepiandrosterone in Human Colonic Adenocarcinoma Cells

in Vitro\textsuperscript{1}

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ABSTRACT

Treatment of mice and rats with the adrenal steroid, dehydroepiandrosterone (DHEA), protects against spontaneous and chemically induced tumors. The mechanism of the chemopreventive action of DHEA, however, remains uncertain. DHEA has been reported to inhibit cholesterol biosynthesis. Mevalonic acid constitutes the basic precursor not only for cholesterol but also for a variety of nonsterol isoprenoids involved in cell growth. Certain of these nonsterol isoprenoids are utilized for posttranslational modification of proteins including p21\textsuperscript{ras}. We therefore investigated the effects of DHEA upon protein isoprenylation. Twenty-four-hour exposure of HT-29 SF human colonic adenocarcinoma cells to 50 \(\mu\)M DHEA was associated with significant incorporation of products of \(^{14}\text{H}\)mevalonate metabolism into several size classes of cellular proteins. The pattern of incorporation was similar to that obtained after treatment with 25 \(\mu\)M lovastatin, a specific 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor. Very little incorporation of label from \(^{14}\text{H}\)mevalonate was observed in untreated cells. This suggests that \(^{14}\text{H}\)mevalonate gains entrance to isoprenylation sites after treatment with DHEA or lovastatin because of depletion of endogenous mevalonate and subsequent inhibition of protein isoprenylation. Isoprenylation plays a critical role in promoting the association of p21\textsuperscript{ras} with the cell membrane. Posttranslational processing and membrane association of p21\textsuperscript{ras} were both found to be inhibited by DHEA. Thus, it is possible that the inhibition of isoprenylation of p21\textsuperscript{ras} and other cellular proteins by DHEA may contribute to its anticancer effects.

INTRODUCTION

DHEA\textsuperscript{3} is a naturally occurring steroid secreted by the adrenal cortex with apparent chemoprotective properties in animal models of tumorigenesis (1, 2). In humans, DHEA serum levels decline markedly with advancing age after peaking in the third decade of life (3, 4). Low serum levels of DHEA have been correlated with an increased risk of developing breast cancer in women (5) or bladder cancer in either sex (6). Administration of DHEA to animals has been found to be protective in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.\textsuperscript{1} This work was supported in part by the Medical Foundation of East Carolina University and by Grant 111 from the Institute of Nutrition of the University of North Carolina at Chapel Hill. S. S. is the recipient of a Technology Transfer Trainee Fellowship from the Biomedical Science Exchange Program between the United States and Germany.

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\textsuperscript{3} The abbreviations used are: DHEA, dehydroepiandrosterone; HMG-CoA reductase, 3-hydroxy-3-methyl-glutaryl-CoA reductase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; G6PDH, glucose-6-phosphate dehydrogenase.

\textsuperscript{1} The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{1} Received 7/1/91; accepted 10/3/91.

MATERIALS AND METHODS

Materials. R-[5-\(^{14}\text{H}\)]Mevalonic acid (30 Ci/mmol), L-[\(^{15}\text{S}\)]methionine (800 Ci/mmol), and Ras 11 anti-ras p21 (pan) antibody were purchased from Du Pont-New England Nuclear (Boston, MA). \(^{14}\text{C}\)-methylated protein molecular weight markers were purchased from Amersham (Arlington Heights, IL). DHEA was purchased from AKZO (Basel, Switzerland). Lovastatin (mevinolin) was prepared as previously described (29) and stored as 10 \(\mu\) stock in dimethyl sulfoxide at \(-20^\circ\text{C}\) until use. Lovastatin was a kind gift from A. W. Alberts (Merck, Sharp and Dohme Research Pharmaceuticals, Rahway, NJ). Lovastatin (mevinolin) was prepared as previously described (29) and stored as 10 \(\mu\) stock in dimethyl sulfoxide at \(-20^\circ\text{C}\). HT-29 human colonic adenocarcinoma cells were purchased from the American Type Culture Collection (Rockville, MD). PC-1 serum-free culture medium was purchased from Ventrex (Portland, ME), and Dulbecco’s modified Eagle’s medium without l-methionine was obtained from Gibco Laboratories (Grand Island, NY). Rabbit anti-mouse IgG antibody and fixed Staphylococcus aureus Cowan I were purchased from Sigma Chemical Co. (St. Louis, MO). The scintillation cocktail used was EcoLite (+) from ICN Biomedicals (Irvine, CA).

Cell Culture. HT-29 SF cells represent a subline of HT-29 cells adapted in our laboratory for growth in completely defined serum-free PC-1 medium. Cells were grown in log phase at 37°C in a humidified
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atmosphere containing 5% CO2. Under these conditions the doubling time for HT-29 SF cells was 24 h.

Growth Inhibition Assay. On day 0, cells were trypsinized, and 104 cells were plated in 60-mm dish in quintuplicate. On day 2, cells were given fresh medium containing either DHEA or lovastatin at concentrations of 0, 5, 25, or 50 μM. Twenty-four h later, the drugs were removed and fresh medium was added. Cells were subsequently allowed to grow until colonies formed. After Giemsa staining the number of colonies of varying size was determined at levels of discrimination of 0.0, 0.2, and 0.4 mm using an Artek model 880 automated colony counter (Farmingdale, NY). By comparing differences in colony diameter, the effects of treatment conditions upon cell growth were quantitated.

Incorporation of [3H]Mevalonate into Cellular Proteins. HT-29 SF cells were plated at a density of 2.5 × 104/100-mm dish. After 2 days cells received either 50 μM DHEA, 25 μM lovastatin, or no treatment for 24 h. Labeling with [3H]mevalonate (82 μCi/ml) was carried out in 4 ml of PC-1 medium during the final 12 h of treatment. Cells were subsequently washed twice with ice-cold phosphate-buffered saline and lysed on ice in detergent buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 25 mM Tris (pH 7.4), 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin (30). After sonication, debris was removed by centrifugation at 100,000 × g for 30 min at 4°C. Protein concentrations were determined by the Bradford reaction (Bio-Rad, Richmond, CA). Equal protein amounts (200 μg) of each sample were subjected to 12.5% SDS-PAGE (31). For quantitative analysis, lanes of interest were sliced into 1-mm fractions with an electrical gel slicer (model 195; Bio-Rad). Gel slices were placed into individual scintillation vials and solubilized (32). Scintillation cocktail was added, and radioactivity was determined using a liquid scintillation spectrometer (Tri-Carb 2200CA; Packard, Downers Grove, IL). The incorporation of radioactivity was expressed as dpm/gel fraction.

Triton X-114 Phase Partitioning of Cell Lysates and Immunoprecipitation of p21*. HT-29 SF cell culture and drug treatments were performed as indicated above. Labeling with [35S]methionine (10 μCi/ml) was carried out in 4 ml of methionine-free Dulbecco’s modified Eagle’s medium during the final 4 h of DHEA or lovastatin exposure. Cells were subsequently washed twice with ice-cold phosphate-buffered saline and lysed on ice in Triton X-114 lysis buffer (1% Triton X-114, 150 mM NaCl, 25 mM Tris, pH 7.4, and protease inhibitors as above). After sonication, insoluble material was removed by centrifugation at 100,000 × g for 30 min at 4°C. The supernatants were transferred into new tubes and phase separated (33). Briefly, after warming at 37°C for 2 min, the turbid solution was centrifuged at 3,000 × g for 5 min at room temperature to yield an upper detergent-depleted phase and a lower detergent-enriched phase. Aliquots of each phase containing 106 trichloroacetic acid-precipitable cpm were immunoprecipitated with 10 μg of mouse monoclonal antibody Ras 11. Following 2 h of incubation with Ras 11 antibody, rabbit anti-mouse IgG antibody was added, and after an additional 1-h incubation, immune complexes were collected using 100 μl of a 10% suspension of fixed S. aureus Cowan I. Immunoprecipitable material was washed three times with Triton X-114 lysis buffer and twice with Tris-buffered saline, boiled in 50 μl SDS sample buffer, and subjected to 15% SDS-PAGE. After soaking in fluorographic enhancer (Amplify; Amersham), gels were exposed to X-Omat AR film for 4 days. Densitometry was performed on the developed film using a scanning densitometer (GS-300; Hoefer, San Francisco, CA).

Subcellular Fractionation and Western Blotting of p21*. HT-29 SF cells were cultured as above. After 24 h of exposure to 50 μM DHEA, cells were washed with phosphate-buffered saline and subsequently scraped into hypotonic lysis buffer (10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 1 mM MgCl2, 1 mM ethyleneglycol-bis(N,N’-tetraacetic acid, and proteinase inhibitors as above) and swollen on ice for 15 min. Cells were homogenized, and debris was removed by centrifugation at 3,000 × g for 10 min at 4°C. The postnuclear supernatant was centrifuged at 100,000 × g for 30 min at 4°C (24). The supernatants were collected and the pelletted membranes were resuspended in detergent buffer. Equal protein amounts (500 μg) of each fraction were subjected to 15% SDS-PAGE and immunoblotted as described (34). Ras proteins were detected with Ras 11 antibody. Blots were developed using a horseradish peroxidase-conjugated anti-mouse antibody (Du Pont).

RESULTS

Growth Inhibition by DHEA: Comparison with Lovastatin. HT-29 SF cells were treated with either DHEA or lovastatin at concentrations of 0, 5, 25, or 50 μM for 24 h (Fig. 1). Both DHEA and lovastatin treatments produced a strong inhibition of cell growth in a dose-dependent manner. While the number of colonies was not reduced by drug treatment at levels of discrimination of 0.0 (data not shown) and 0.2, the number of colonies enumerated at the 0.4-mm discrimination level was decreased, indicating that both DHEA and lovastatin inhibited cell growth but not cell viability under these conditions. Administration of 5, 25, or 50 μM DHEA inhibited cell growth to 85%, 73%, and 47% of control levels, respectively. Administration of 5, 25, or 50 μM lovastatin inhibited cell growth to 79%, 65%, and 38% of control levels, respectively. It has been shown that 15–30 μM lovastatin completely inhibits endogenous mevalonate synthesis and isoprenylation-dependent protein maturation in a variety of cultured cells (20, 35–38). For this reason, and because 50 μM DHEA produced an effect upon cell growth not significantly different from that produced by 25 μM lovastatin, we used these drug concentrations in our subsequent studies of protein isoprenylation.

DHEA Inhibits Protein Isoprenylation. HT-29 SF cells were exposed to 50 μM DHEA or 25 μM lovastatin for 24 h with [3H]mevalonate added during the final 12 h of drug treatment. When 200 μg of protein extracted from these cells were subjected to SDS-PAGE, the resulting gel was sliced into 1-mm fractions, and the fractions were quantitated for proteins incorporating products of [3H]mevalonate metabolism. Significant incorporation of label into several size classes of proteins was observed in DHEA- and lovastatin-treated (but not control) cells (Fig. 2). Specific peaks of radioactivity were observed at molecular weights of 14,000–18,000, 20,000–30,000, and 46,000–69,000. The pattern of incorporation of label from [3H]mevalonate into proteins in DHEA-treated cells was essentially the same as that of lovastatin-treated cells.

Fig. 1. Inhibition of cell growth by DHEA and lovastatin. HT-29 SF cells were treated for 24 h with either DHEA or lovastatin at concentrations of 0, 5, 25, or 50 μM and subsequently allowed to grow until colonies formed (72 h). Effects of treatment conditions upon cell growth were determined by comparing the number of colonies at 0.2 and 0.4 mm levels of discrimination using an automated colony counter. Points, numbers of colonies as the percentage of untreated controls; bars, SE. The number of colonies ± SEM in untreated controls at levels of discrimination of 0.2 mm and 0.4 mm were 845 ± 18 and 154 ± 21, respectively. The doubling time of HT-29 SF cells was 24 h. Each data point was performed in quintuplicate, and the experiment was repeated three times.

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sample were subjected to SDS-PAGE, and the resulting gel was sliced. Gel slices were treated with [3H]mevalonate. Equal amounts of protein (200 µg) from each sample were subjected to SDS-PAGE, and the resulting gel was sliced. Gel slices (1 mm) were solubilized and assayed for radioactivity. A, top, migration of protein molecular weight markers (M, x 10^3). B, from cells treated for 24 h with 25 µM lovastatin or not treated (control). Incorporation of products of [3H]mevalonate metabolism was determined as in A. Representative results are from one of four independent experiments performed using the same method.

Fig. 2. Effects of DHEA and lovastatin on the incorporation of products of [3H]mevalonate metabolism into cellular proteins. A, from cells treated for 24 h with 50 µM DHEA or not treated (control) and labeled during the final 12 h of treatment with [3H]mevalonate. Equal amounts of protein (200 µg) from each sample were subjected to SDS-PAGE, and the resulting gel was sliced. Gel slices (1 mm) were solubilized and assayed for radioactivity. A, top, migration of protein molecular weight markers (M, x 10^3). B, from cells treated for 24 h with 25 µM lovastatin or not treated (control). Incorporation of products of [3H]mevalonate metabolism was determined as in A. Representative results are from one of four independent experiments performed using the same method.

DISCUSSION

Our results suggest strongly that DHEA, in a manner analogous to that observed by several other laboratories for the HMG-CoA reductase inhibitor lovastatin (17, 19, 27, 37, 38), depletes intracellular mevalonate pools under in vitro conditions.

Fig. 3. SDS-PAGE analysis of the effects of DHEA and lovastatin on the posttranslational processing of p21ras. Cells were treated for 24 h with either 50 µM DHEA or 25 µM lovastatin (LOV) or were not treated (CON) and were labeled with [35S]methionine during the last 4 h of drug exposure. Cells were then lysed in Triton X-114, and the lysates were partitioned into a detergent-depleted aqueous phase (a) and a detergent-enriched phase (d). Aliquots of each phase containing 10^6 trichloroacetic acid-precipitable cpm were immunoprecipitated and resolved on SDS-PAGE, and the presence of p21ras was detected by fluorography. Arrow, position of p21ras. Ordinate, migration of protein molecular weight markers (M, x 10^3).
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Fig. 4. Western blot analysis of the effect of DHEA on the membrane localization of p21ras. Cells were treated for 24 h with 50 μM DHEA or were not treated (CON). Cell lysates were separated by centrifugation into soluble (S) and particulate (P) fractions. The samples were then resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, and p21ras was detected with Ras 11 antibody. Arrow, position of p21ras. Ordinate, migration of protein molecular weight markers (M, x 10^3). Representative results are from one of five independent experiments performed using the same method.

Mevalonate is the central regulated precursor for cholesterol biosynthesis but also serves as a precursor for a variety of nonsterol substances essential for cell growth, including farnesyl-PP, and geranylgeranyl-PP (40–43). Farnesyl-PP, and geranylgeranyl-PP, represent the immediate precursors for protein isoprenylation reactions, a type of posttranslational modification which enhances the hydrophobicity of proteins. Such isoprenylation events often precede additional posttranslational modifications and are apparently a necessary prerequisite for membrane association of at least some proteins (reviewed in Ref. 16). Cholesterol biosynthesis has been shown to be inhibited after DHEA treatment (14, 15), and we now report that the isoprenylation of cellular proteins, including p21ras, is also inhibited by exposure to DHEA. Furthermore, the DHEA-mediated inhibition of p21ras isoprenylation was observed to prevent p21ras association with the plasma membrane. Inasmuch as oncogenic ras proteins are associated with the development of a wide array of human and animal tumors (reviewed in Ref. 21), the ability of DHEA to deplete intracellular mevalonate pools and inhibit p21ras isoprenylation and membrane association may contribute to the anticancer activity of this steroid in some tumor types.

Unlike Lovastatin, which is a direct competitive inhibitor of HMG-CoA reductase, DHEA is apparently pleiotropic in its effects upon cellular metabolism. Our data, for example, indicate that, while 50 μM DHEA produced only about 40% of the effect of 25 μM Lovastatin upon protein isoprenylation, it was equally effective in inhibition of HT-29 SF cell growth. This result suggests that the growth-inhibitory effect of DHEA may include additional mechanisms other than depletion of mevalonate pools and inhibition of protein isoprenylation. DHEA is a potent uncompetitive inhibitor of mammalian G6PDH (44), the rate-limiting enzyme in the hexose monophosphate shunt. Two potential consequences of this inhibition are restriction on the availability of NADPH and of 5-carbon sugars, both of which are required for synthesis of purine and pyrimidine nucleotides (45). Nucleotide pool depletion may therefore contribute to the growth-inhibitory effects of DHEA but would not be expected during growth inhibition induced by Lovastatin (11, 46).

HMG-CoA reductase is an unusual enzyme of intermediary metabolism in that it requires 2 mol of NADPH for each mole of product (mevalonate) produced. Since the hexose monophosphate shunt represents a major source of extramitochondrial NADPH, and since DHEA is a potent uncompetitive inhibitor of the rate-limiting enzyme (G6PDH) of this pathway, an attractive hypothesis is that DHEA-mediated inhibition of protein isoprenylation occurs due to depletion of NADPH cofactor necessary for HMG-CoA reductase activity. However, at least in hepatic cells, malic enzyme is apparently capable of providing sufficient NADPH to prevent levels from falling below normal (14). Thus, it remains possible that DHEA’s effects upon protein isoprenylation may be mediated in a manner not related to depletion of NADPH cofactor in HT-29 SF cells. However, indirect evidence that NADPH levels may in fact be sufficiently reduced in DHEA-treated cells to create a biological effect comes from work showing that the metabolism of aflatoxin B1 and of 7,12-dimethyl-benz[a]anthracene by cytochrome P450, an NADPH-requiring mixed-function oxidase enzyme, is inhibited in DHEA-treated cells in vitro (47). Furthermore, the possibility exists of intracellular compartmentalization of NADPH pools with the result that biochemical estimations of total NADPH levels may not reflect local concentrations of this cofactor at the site of mevalonate biosynthesis, carcinogen metabolism, or other NADPH-dependent processes.

If DHEA’s inhibitory action upon protein isoprenylation is mediated via NADPH cofactor depletion, then any steroid capable of inhibiting G6PDH would be expected to have similar effects upon protein isoprenylation. For example, in addition to DHEA, other steroids in the androstane series, as well as pregnenolone and related pregnane steroids and certain fluoro-substituted and bromine-substituted analogues of DHEA, are also known to inhibit G6PDH (reviewed in Ref. 1). However, no inhibition of protein isoprenylation has been observed following treatment of mammalian cells with either dexamethasone (38) or 25-hydroxycholesterol (27), indicating that this effect of DHEA is not a general property of the steroid nucleus.

In summary, our findings suggest that DHEA depletes intracellular mevalonate pools, inhibits protein isoprenylation, and blocks p21ras posttranslational processing and membrane association. These studies represent an attempt to understand the anticancer action of DHEA on the molecular level.

ACKNOWLEDGMENTS

The authors are grateful to A. W. Alberts of Merck, Sharp and Dohme Research Laboratories for his generous supply of Lovastatin, to Drs. Richard Klann and So Chun Wong for a critical review of the
manuscript, and to Solveig Schönfeld, Dawn Canupp, and Sherry Leonard for their excellent technical assistance. The support and encouragement of Dr. C. Tate Holbrook are also gratefully acknowledged.

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