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Cholesterol Inhibits the Nuclear Entry of Estrogen Receptor Activation Factor (E-RAF) and Its Dimerization With the Nonactivated Estrogen Receptor (naER) in Goat Uterus

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Abstract An alternative form of estrogen receptor isolated from goat uterus, the nonactivated estrogen receptor (naER), has no DNA-binding function, although it is closely similar to the classical estrogen receptor (ER) in its hormone binding affinity and specificity. The naER dimerizes with a DNA binding protein, estrogen receptor activation factor (E-RAF). The heterodimer binds to the DNA. Assays carried out during the purification of E-RAF showed that an endogenous inhibitor that is heat stable and dialyzable bound to the E-RAF and prevented the formation of the heterodimer. The inhibitor has been isolated and purified. GC-MS analysis identifies this molecule to be cholesterol. Circular dichroism measurement has shown that the high-affinity binding of cholesterol to E-RAF results in subtle changes in the secondary and the tertiary structure of the protein. The E-RAF with altered conformation fails to dimerize with the naER. Instead of facilitating E-RAF entry into the nucleus, dimerization with the naER prevents it. Similarly, cholesterol binding blocks the nuclear entry of the protein, showing that E-RAF with altered conformation is incapable of interaction with the nuclear pore complex/membrane proteins. The naER-E-RAF heterodimer remains at the nuclear periphery, incapable of further transport. These results indicate the possibility that the dimerization between naER and the E-RAF takes place only within the nuclear compartment. The observation that cholesterol binding prevents nuclear entry of the E-RAF reflects the similarity of E-RAF with the sterol regulatory element (SRE) binding protein that enters the nucleus and binds to SRE only when the intracellular level of cholesterol remains low. J. Cell. Biochem. 77: 382–395, 2000.

Key words: cholesterol; estrogen receptor activation factor; goat uterus; nonactivated estrogen receptor; nuclear migration

The existence of estrogen receptor activation factor (E-RAF) in the mammalian uterus was demonstrated a decade ago [Thampan and Clark, 1981; Thampan, 1987, 1989]. E-RAF was observed to dimerize with an alternative form of estrogen receptor, identified as the nonactivated estrogen receptor (naER) [Anuradha et al., 1994; Karthikeyan and Thampan, 1996]. The naER is incapable of binding to DNA on its own. Other features in which naER differs from the classical or regular estrogen receptor (ER) are (1) plasma membrane localization of the naER, (2) tyrosine kinase activity associated with the naER, (3) glycoprotein nature of the naER, and (4) display of CNBr peptides

Abbreviations used: E_2, estradiol-17β; naER, nonactivated estrogen receptor, ER, estrogen receptor; E-RAF, estrogen receptor activation factor; GC-MS, gas chromatography-mass spectrometry; NBS, N-bromosuccinimide; FITC, fluorescein isothiocyanate; SRE, sterol regulatory element; LDL, low-density lipoprotein; hsp-90, heat shock protein 90; DCC, dextran-coated charcoal; PEG, polyethylene glycol.

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different from those of the ER. It was long assumed that E-RAF was the mediator involved in the transport of naER to the nucleus, and vice versa. By contrast, the observations presented in this report demonstrate that entry of E-RAF into the nucleus is independent of any other cytosolic protein.

During the purification of E-RAF [Thampan, 1987], it was observed that a heat-resistant and dialyzable endogenous inhibitor prevented DNA binding of the naER-E-RAF complex. The E-RAF assay involved incubation of ^{3}H-estradiol-naER complex with E-RAF and DNA cellulose and the subsequent measurement of the labeled estradiol bound to DNA cellulose. A decline in the hormone bound to the DNA cellulose signaled the absence of a functional E-RAF-naER complex. The observation that the putative "inhibitor" did not interfere with the DNA-binding function of E-RAF suggested that the inhibition manifested at the level of E-RAF-naER dimerization.

We have carried out studies designed to purify and identify this inhibitor. The inhibitor molecule has been identified as unmetabolized cholesterol. The observation raises two possibilities: (1) an apparent role for E-RAF in cholesterol homeostasis, or (2) a regulatory role for cholesterol in E-RAF-mediated events in estrogen action.

MATERIALS AND METHODS

[2,4,6,7-^{3}H]estradiol-17β (spec act 101 Ci/mmol) and [1α,2α (n)^{3}H] cholesterol (spec act 47 Ci/mmol) were purchased from Amersham. Nonradioactive estradiol-17β-diethylstilbestrol (DES), p-aminobenzamidine agarose phenylmethylsulfonyl fluoride (PMSF), and fluorescein isothiocyanate were purchased from Sigma Chemical Co. Nonradioactive cholesterol was purchased from E-Merck (Germany). DE-52, phosphocellulose, and cellulose CF-11 were obtained from Whatman (England), and silica gel G from BDH. Sepharose 4B was obtained from Pharmacia. Hydroxyapatite (HAP) was purchased from Bio-Rad. Routine chemicals of analytical or reagent grade were purchased from local commercial establishments. Synthetic derivatives of cholesterol used in the competitive binding assays were obtained from Aldrich.

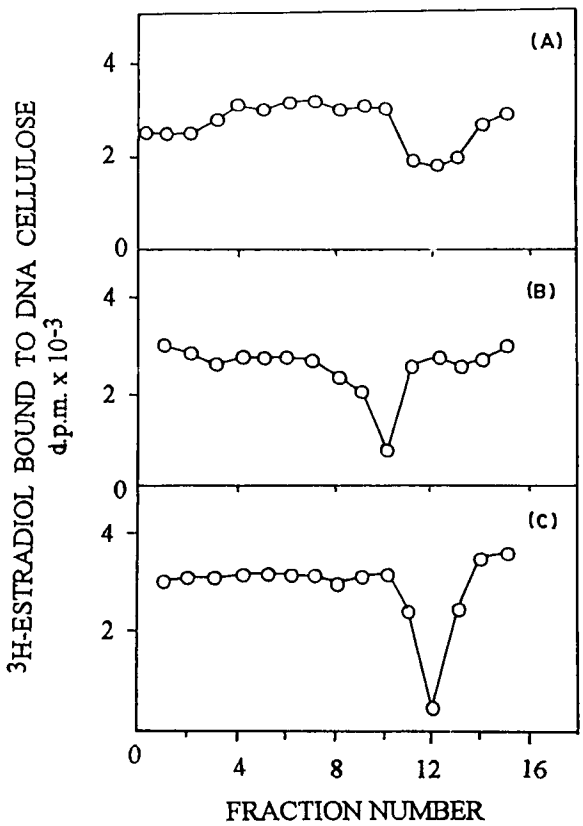

Fig. 1. Thin-layer chromatographic (TLC) analysis of the DE-52 bound inhibitor. The DE-52 bound fraction with inhibitor activity was subjected to TLC using three different solvent systems. It was first subjected to TLC in (**A**) a solvent system containing butanol-acetic acid-water (70:15:15). The active fraction from this was subjected to TLC (2) (**B**) using hexane-ethyl acetate (1:1) as the solvent system. The active component from this system was subjected to TLC (3) (**C**) with a solvent system containing chloroform-methanol (1:1). The gel area, partitioned into 1-cm squares, was extracted with 2:1 chloroform-methanol. The extract was dried in assay tubes before its exposure to the assay constituents.

Purification of Estrogen Receptor Activation Factor (E-RAF)

The method developed earlier [Thampan, 1987, 1989] was followed in the current study.

Purification of Nonactivated Estrogen Receptor (naER)

Goat uterine naER was isolated and purified following the method published earlier from our laboratory [Anuradha et al., 1994; Karthikeyan and Thampan, 1996].

Extraction and Partial Purification of the Endogenous Inhibitor from Goat Uterus

Goat uteri were obtained from a local slaughterhouse. The tissue was homogenized in

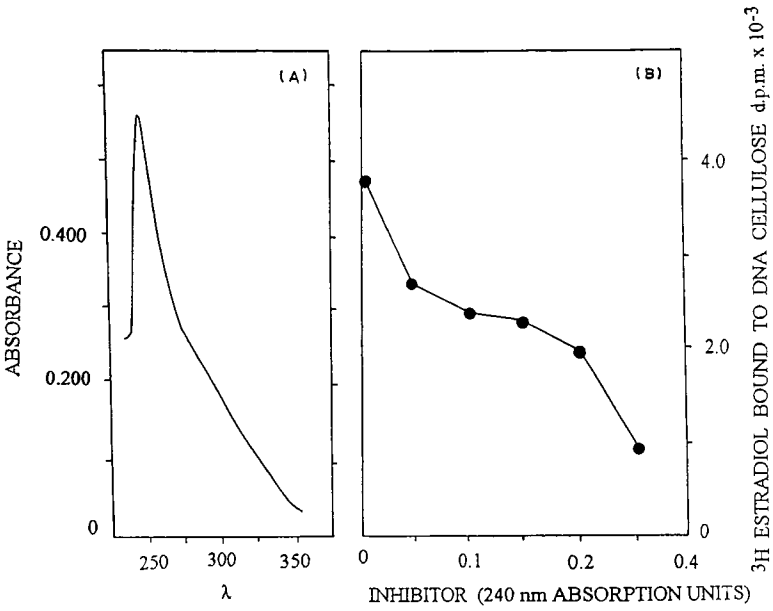

Fig. 2. **A:** UV spectrum of the partially purified inhibitor obtained at the end of thin-layer chromatography (TLC) (3). The molecule displayed an absorption maximum at 243 nm. **B:** DNA cellulose binding assay showing the effect of the partially purified inhibitor. The assay method has been given under Materials and Methods. ^{3}H-estradiol- nonactivated estrogen receptor (naER) complex was incubated with estrogen receptor activation factor (E-RAF) and DNA-cellulose in the presence of varying concentrations of the inhibitor obtained from TLC (3). The ^{3}H-E_2 bound to the DNA at the end of the incubation, was measured.

TEMN buffer (10 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, 12 mM monothioglycerol, and 50 mM NaCl), using an Ultra Turrax homogenizer. The homogenate was heated in a boiling water bath for 30 min, after which it was centrifuged at 15,000*g* for 15 min. The coagulated material was discarded. The supernatant was mixed for 30 min with activated charcoal (0.1 g/ml). The charcoal pellet was recovered after centrifugation and extracted with chloroform-methanol (2:1). The chloroform-methanol extract was dried under an airstream, after which the dried material was dissolved in TEMN buffer containing 10% ethanol. The extract was chromatographed over a column of Whatman DE-52 equilibrated with TEMN buffer. The inhibitor assay carried out using the DE-52 fractions showed that the factor was bound to the matrix. The material that was eluted from the column using 1 M NaCl was subjected to the purification protocol.

Thin-Layer Chromatographic Analysis

The DE-52 fractions that displayed the inhibitor activity were extracted with chloroform-methanol (2:1). The extracted material was dried and redissolved in the organic solvent used as the mobile phase in the first of the three thin-layer chromatography (TLC) (silica gel G) systems followed: (1) butanol-acetic acid-water (70:15:15), (2) hexane-ethyl acetate (1:1), and (3) chloroform-methanol (1:1). The gel area was partitioned and scraped out. Each gel sample was extracted with chloroform-methanol (2:1), after which the extract was dried and assayed for the inhibitory activity. The active fraction from TLC (1) was subjected to analysis in TLC (2) and that of TLC (2) was analyzed in TLC (3). The active fraction obtained from TLC (3) was subjected to gas chromatography-mass spectrometry (GC-MS) analysis.

Preparation of DNA Cellulose

The nature of the DNA-binding site of the E-RAF is unknown. Consequently, we had to depend on the traditional DNA cellulose-binding assay in detecting naER-E-RAF dimerization, using mammalian DNA immobilized on Whatman CF-11. Goat uterine or liver DNA was extracted as described by Marmur [1961] and immobilized on Whatman CF-11 following the method of Alberts and Herrick [1971].

Inhibitor Assay

The active fraction derived from the three TLC systems was collected in assay tubes and dried under a stream of air. Nonactivated estrogen receptor (naER), isolated and purified from the goat uteri, was incubated overnight with 20 nM ^{3}H-estradiol, at 4°C. The unbound hormone was removed after adsorption to dextran-coated charcoal (1% activated charcoal and 0.1% dextran suspended in 10 mM Tris-HCl, pH 7.6, containing 10% glycerol). The ^{3}H-

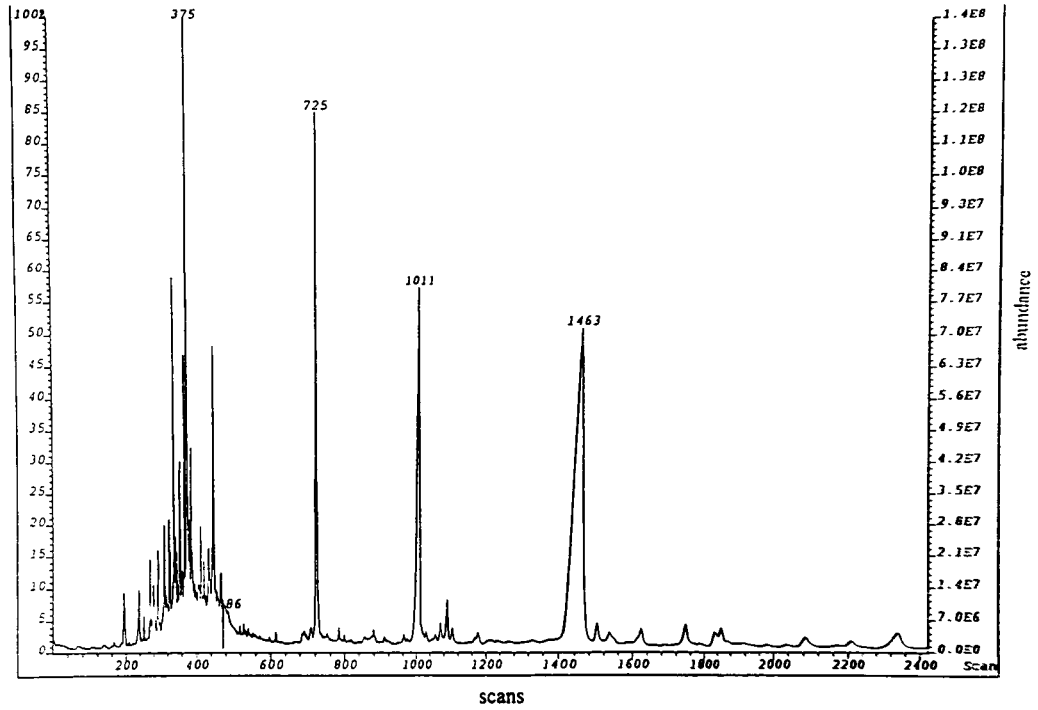

Fig. 3. Gas chromatography-mass spectrometry (GC-MS) analysis of the inhibitory material obtained at the end of TLC (3). The peaks selected for further analysis were those that appeared at scans 1463 and 1011, as well as the cluster observed between 200 and 800 scans. The peaks observed at 725 and 375 scans were due to phthalate impurity.

estradiol-naER complex was mixed with an equimolar concentration of E-RAF and added to assay tubes containing the dried inhibitor material. The mixture was incubated with DNA cellulose at 30°C for 30 min. The reaction was stopped after the addition of 2 ml ice cold TEMN buffer to each tube. The samples were centrifuged and the supernatants discarded. The DNA-cellulose pellets were washed twice with ice cold TEMN buffer. The washed pellet was extracted with 1 ml distilled ethanol. The radioactivity associated with the extract was measured using a Wallac 1409 liquid scintillation counter.

Mass Spectrometry

Mass spectral analysis was done with VG AUTOSPEC-M mass spectrometer with the OPUS V3 IX data system. The following conditions were maintained during the analysis. The source temperature was 200°C, the trap current 200 A, and Electron energy 70 eV.

Gas Chromatography-Mass Spectrometry

GC-MS analysis was carried out on the same instrument, using the following parameters. The column used was OV-1 fused silica capillary column of 30 mm length, 0.31-mm inner diameter (ID) and 0.25-μm film thickness, with an initial temperature set at 100°C for 5 min and a final temperature at 200°C for 90 min. The ramp rate of heating was set at 10°C/min.

Synthesis of Cholesta-4,6-Diene-3-ol

A method developed by Tachibana [1986] was followed. A hexane solution of cholesteryl acetate (2.48 g), N-bromosuccinimide,1.77 g (NBS), and a catalytic amount of benzoyl peroxide was allowed to react under reflux for 40 min. The hexane solution was filtered to remove succinimide and the filtrate was evaporated to dryness under reduced pressure below 40°C. The residue was dissolved in butyl acetate (8 ml). Tetrabutylammonium bromide (0.346 g) and 2,4,6-trimethyl pyridine (1.45 g) were added to the butylacetate solution. The mixture was allowed to reflux for 20 min. The solution was washed with dilute HCl and water, dried over sodium sulfate, and finally concentrated to a semicrystalline residue under reduced pressure. To the residue methanolic KOH solution (KOH 0.4 g, MeOH 10 ml) was

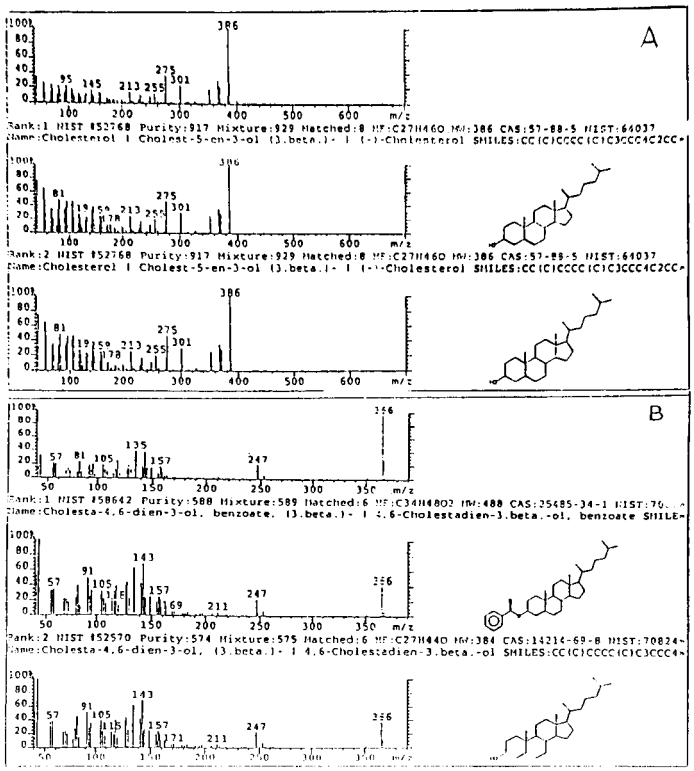

Fig. 4. Mass spectra of the gas chromatography (GC) peaks and computer search results of the mass spectrometry (MS) data. The GC 1463 scan peak in Fig. 4 has been identified as cholesterol while the peak at GC scans 1011 has been identified as cholesta-4,6-diene-3-ol or its benzoate derivative.

added and the mixture was allowed to stand overnight at room temperature. The crude cholesta-4,6-diene-3-ol was collected by filtration and was purified by chromatography on silica gel. The synthesized compound was checked for its purity through MS.

Sucrose Density Gradient Analysis

^{3}H-estradiol-naER complex was incubated overnight with E-RAF and the inhibitor at 4°C, after which the samples were subjected to sucrose density gradient centrifugation (linear gradients of 5–20% sucrose in TEM buffer containing 0.3M NaCl) for 16 h at 55,000 rpm in a SW 65 rotor of a Beckman ultracentrifuge. The fractions collected were analyzed for radioactivity. Human gamma globulin and bovine serum albumin (BSA) were used as sedimentation markers.

Nuclear Binding of the naER-E-RAF Heterodimer

Goat uterine nuclei were isolated following a method described earlier [Thampan, 1985], except that the nuclei were not treated with Triton × 100. The isolated nuclei were suspended in TMKC-sucrose buffer (50 mM Tris-HCl, pH 7.6, containing 2 mM $MgCl_2$, 20 mM KCl, 1 mM $CaCl_2$, 0.2 mM PMSF, and 250 mM sucrose). The naER was incubated overnight at 0–4°C with 20 nM ^{3}H-estradiol, and the unbound hormone was removed after DCC treatment, as described earlier. The nuclei, suspended in TMKC-sucrose buffer containing 4 mM ATP, were incubated with ^{3}H estradiol-naER complex, E-RAF and the inhibitor at 30°C for 30 min. The incubated samples (250 μl) were layered over 10-ml pads of TMKC buffer containing 0.34 M sucrose and centrifuged at 1,000*g* for 10 min in a Sorwall RC-5B centrifuge. The nuclear pellet collected at the bottom of the tube was extracted with 1 ml ethanol. The radioactivity associated with the ethanol extract was measured using a toluene-based and Triton X-100 containing scintillation medium.

Saturation Binding of ^{3}H-Cholesterol to E-RAF

E-RAF, purified to homogeneity, was incubated with ^{3}H cholesterol (1–20 nM) in the

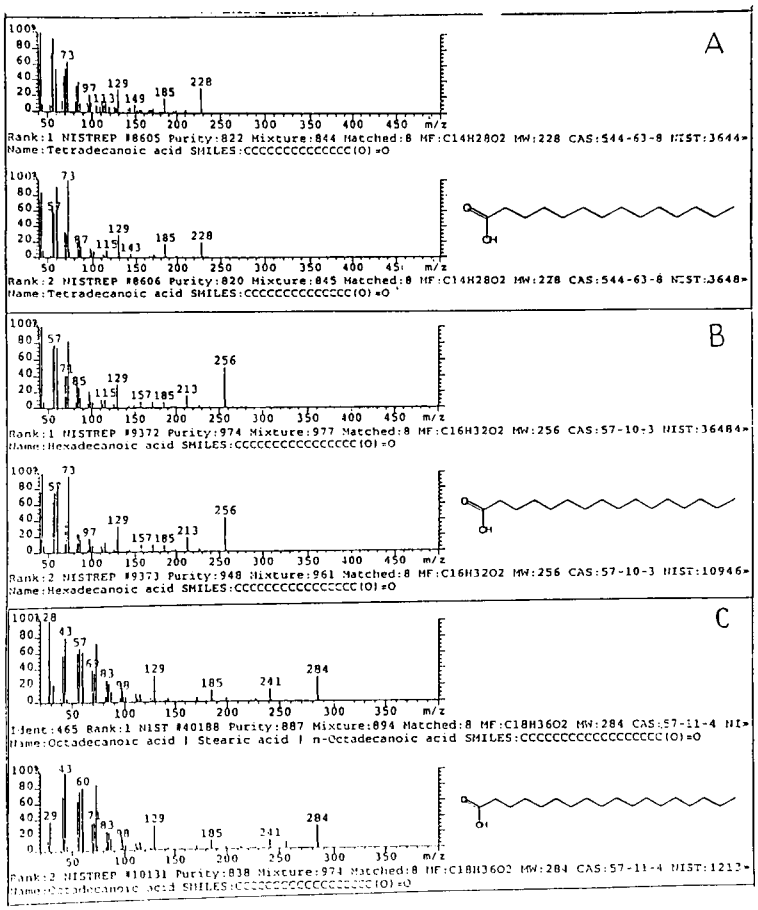

Fig. 5. Mass spectra and the computer search data of the fatty acids located in the peak cluster observed between 200 and 800 scans in the gas chromatography (GC) trace. **A:** Myristic acid. **B:** Palmitic acid. **C:** Stearic acid.

presence or absence of a hundred fold excess of unlabeled cholesterol overnight at 4°C. The specific binding was calculated after a hydroxyapatite (HAP) adsorption assay [Clark and Peck, 1971].

Circular Dichroism Measurement

Interaction of cholesterol with E-RAF was subjected to circular dichroism (CD) analysis, using a Jasco Spectropolarimeter. Scans were made at a wavelength range of 330–240 nm (0.5 sensitivity) in order to detect changes in the tertiary structure and also at 250–210 nm (2.0 sensitivity), in order to identify changes in the secondary structure of the E-RAF.

Nuclear Transport of FITC-Labeled Proteins

FITC-labeled naER or E-RAF was incubated with isolated nuclei suspended in TMKC sucrose buffer containing 4 mM ATP at room temperature for 30 min, and the nuclear entry of the proteins was monitored using a Nikon Eclipse E 600 fluorescence microscope.

RESULTS

Thin-Layer Chromatography of the Inhibitor

Three different solvent systems were used in the successive TLC isolation of the inhibitor originally eluted from the DE-52 column: (1) butanol-acetic acid-water (70:15:15), (2) hexane-ethyl acetate (1:1), and (3) chloroform-methanol (1:1). After chromatography, the gel area was fractionated and subsequently extracted with chloroform-methanol (2:1). The extract that was collected and dried in assay tubes was subjected to the inhibitor assay. An enrichment in the inhibitor activity was apparent as the test material passed through the three TLC systems (Fig. 1).

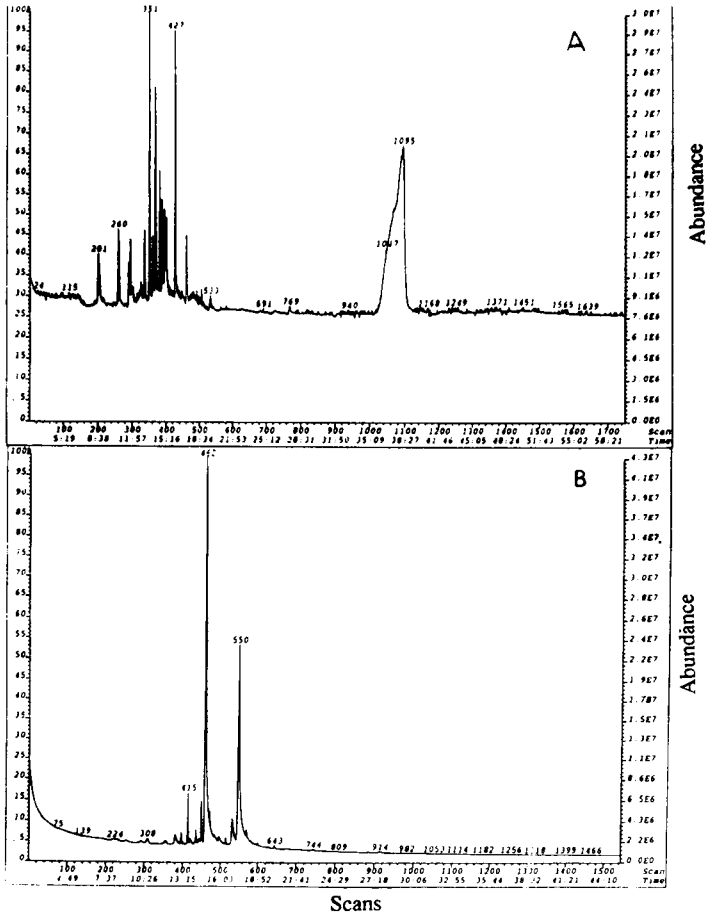

Fig. 6. Gas chromatography-mass spectrometry (GC-MS) analysis of the active factor associated with the immunoprecipitates of estrogen receptor activation factor (E-RAF) and nonactivated estrogen receptor (naER). Purified E-RAF and naER were incubated separately with the partially purified inhibitor, after which the proteins were immunoprecipitated with the corresponding antibody. Solvent extracts of these immunoprecipitates were dried and subjected to GC-MS analysis. **A:** E-RAF immunoprecipitate. **B:** naER immunoprecipitate. The peaks in **A** that appeared at scans 1095, as well as those located between 300 and 550 scans were subjected to mass spectral analysis.

Structural Analysis of the Active Fraction Recovered From TLC (3)

The fraction that contained the inhibitory activity could be identified under an ultraviolet (UV) source due to the high fluorescence displayed by the material. The gel area covered by the fluorescent spot was scraped out and extracted with chloroform.

The inhibitory material partially purified through the three TLC systems was subjected to UV spectroscopy. It was shown to have an absorption maximum at 243 nm (Fig. 2A). An inhibitor assay that was carried out using this partially purified material showed a direct relationship between the inhibitory activity and the concentration of this material in the assay system (Fig. 2B).

GC analysis displayed peaks ranging between 200 and 2,400 scans (Fig. 3). The most prominent among them were the peaks with retention times around 37.8 min (scan 1011) and 53.8 min (scan 1463).

Mass spectra of the GC peaks of 1463 and 1011 scans indicated that they belonged to molecules with molecular mass 386 and 366, respectively (Fig. 4). A library search carried out to identify these compounds indicated that while the mass 386 peak was found to represent cholesterol, the mass 366 peak was shown to represent either cholesta-4,6-diene-3-ol benzoate or cholesta-4,6-diene-3-ol (Fig. 4). However, one would expect the retention time of cholesta-4,6-diene-3-ol to be close to that of cholesterol. Therefore, the observed retention

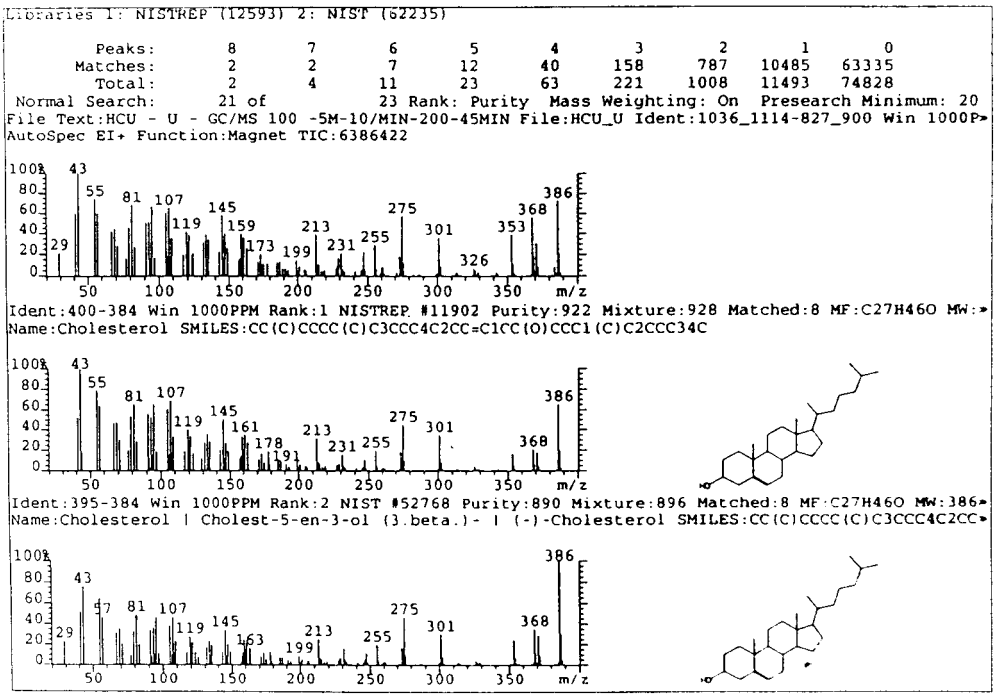

Fig. 7. Mass spectral analysis of the peak that appeared at 1095 scans in the GC of the solvent extracts of estrogen receptor activation factor (E-RAF) immunoprecipitate (see Fig. 6).

time for the scan 1011 suggested that it could be a nonpolar cholestatriene molecule with a possible precursor as cholesta-4,6-diene-3-ol, since the mass spectra of the latter matched closely with that for scan 1011.

A cluster of peaks appeared between 200 and 800 scans. Of these, three peaks were identified as fatty acids corresponding to molecular weights 228, 256, and 284 (Fig. 5). The mass spectra of these compounds matched well with the mass spectra of myristic acid, palmitic acid, and stearic acid in the library search program. The other peaks in the 200- to 600-scan range were identified as artifacts due to phthallates and some hydrocarbons.

Identification of the "Receptor" Protein for the Inhibitor

In order to find out which one of the components identified in the computer search was the true inhibitor, an indirect experimental approach was undertaken. Purified E-RAF and naER were incubated overnight at 4°C with the inhibitor that was isolated through TLC (3). The proteins were subsequently immunoprecipitated using the corresponding (anti naER or anti E-RAF) polyclonal antibody and goat anti rabbit IgG. The immunoprecipitate was washed extensively with TEMN buffer. The precipitate was extracted with chloroform-methanol (2:1) and the extract was subjected to GC-MS analysis. The gas chromatogram of the solvent extract of the E-RAF immunoprecipitate showed a prominent peak at 1095 scans and a cluster of minor peaks between 300 and 500 scans (Fig. 6A). The extract of the naER immunoprecipitate displayed three peaks within the range of 400–550 scans. The 1095-scan peak was conspicuously absent (Fig. 6B).

The mass spectrum of the 1095-scan peak confirmed that it represented cholesterol (Fig. 7). The minor peaks that appeared between 300 and 500 scans were shown to represent palmitic and stearic acids (Fig. 8). Because the peak representing cholesterol was not detected in the naER immunoprecipitate, no effort was made to characterize the peaks that appeared at scans 415, 452, and 550.

Direct Identification of the Inhibitor as Cholesterol

In order to find out the true identity of the inhibitor, it was essential to carry out assays using the molecules identified in the mass spectra computer search. Cholesta-4,6-diene-3-ol was synthesized in the laboratory, while

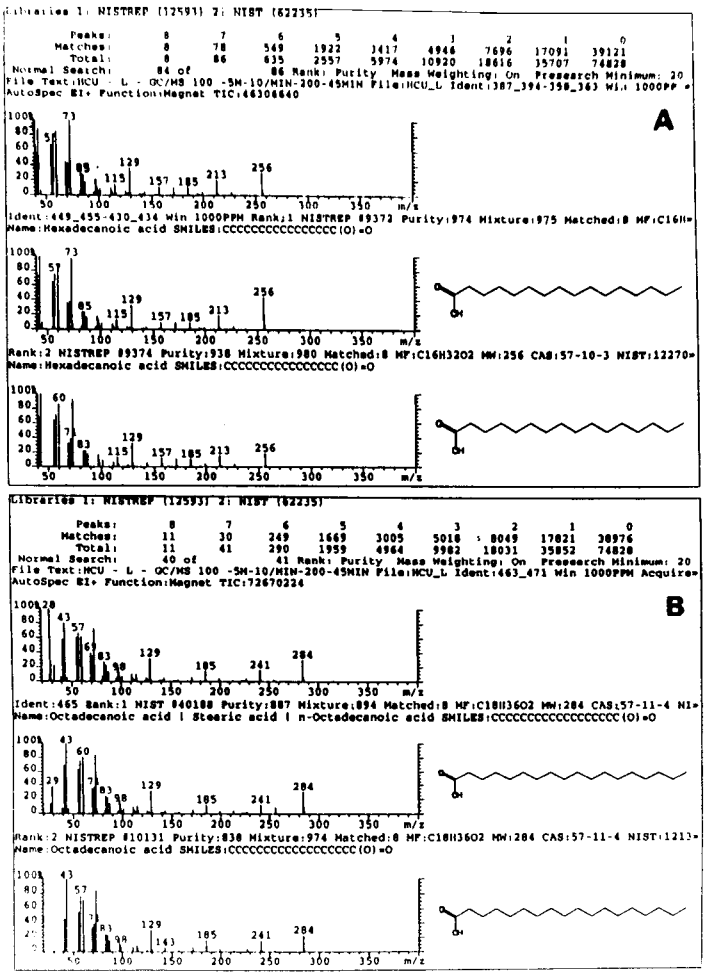

Fig. 8. Mass spectral analysis of the peaks that appeared at scans in the range 300–550 in the gas chromatography (GC) of the solvent extracts of E-RAF immunoprecipitate (see Fig. 6). **A:** Peak at 363 scans. **B:** Peak at 463 scans.

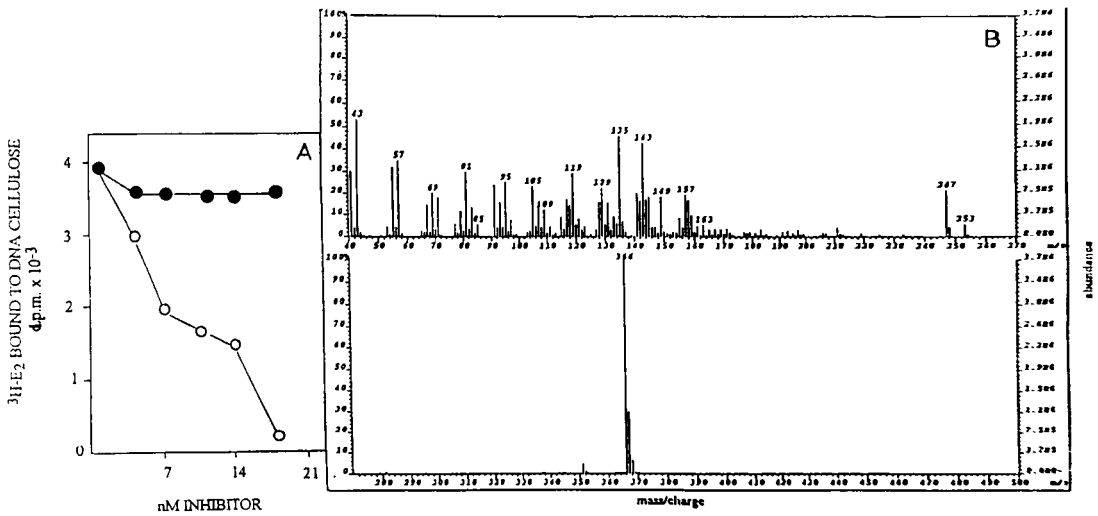

Fig. 9. Analysis of the functional identity of cholesterol and cholesta-4,6-dien-3-ol. **A:** A DNA-cellulose binding assay was carried out using assay systems containing either cholesterol (○) or its metabolite, cholesta-4, 6-diene-3-ol (●). The inhibitor activity is clearly shown here to be associated with cholesterol. **B:** Mass spectrum of the cholesta-4,6-diene-3-ol synthesized within the laboratory and used in the assay system of (A).

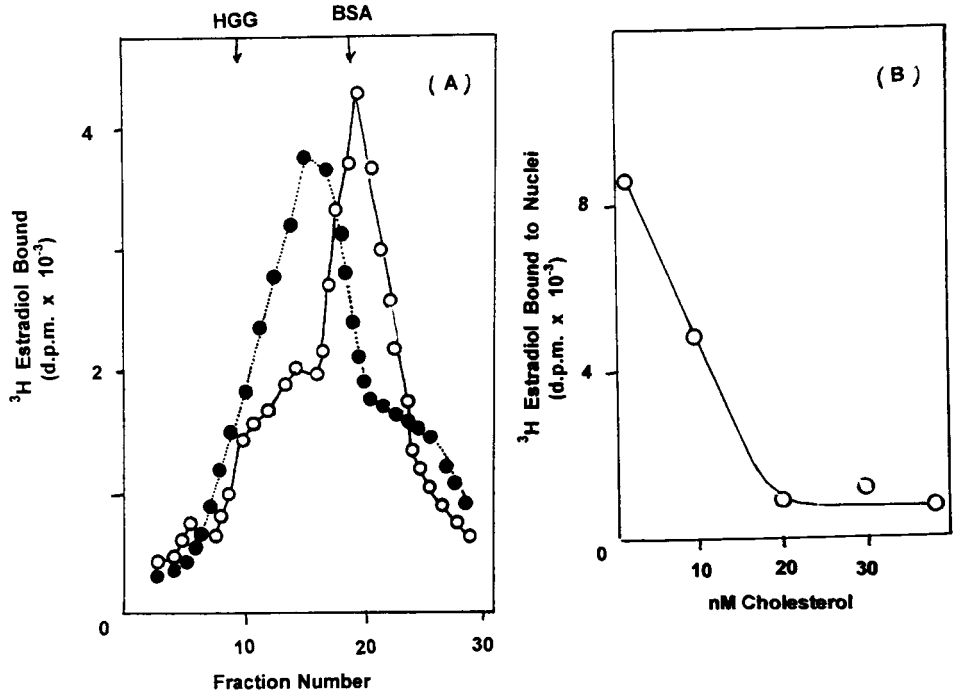

Fig. 10. Functional studies involving cholesterol. **A:** ^{3}H-E_2 nonactivated estrogen receptor (naER) complex and estrogen receptor activation factor (E-RAF) incubated in either the presence (○) or absence (●) of 20 nM cholesterol was subjected to sucrose density gradient analysis. The mixture was layered over 5–20% sucrose density gradients and subjected to centrifugation for 16 h at 55,000 rpm in a SW 65 rotor of the Beckman ultracentrifuge. Fractions collected were analyzed for radioactivity. **B:** Effect of cholesterol on the nuclear binding of ^{3}H-E_2-naER-E-RAF complex. Isolated nuclei were incubated with ^{3}H-E_2-naER and E-RAF in the presence of varying concentrations of cholesterol at 30°C for 30 min. The incubated samples were layered over 10 ml 0.34 M sucrose pads and centrifuged at 1,000*g* for 10 min, as described under Materials and Methods. The radioactivity associated with the nuclei was measured.

the fatty acids and cholesterol were purchased from commercial establishments. DNA-cellulose binding of ^{3}H-E_2 naER was examined in a system containing E-RAF and varying concentrations of these synthetic agents. Cholesterol alone was shown to function as the inhibitor. The maximum inhibition was observed in the presence of <21 nM cholesterol (Fig. 9A). Cholesta-4,6-diene-3-ol was totally ineffective. None of the three fatty acids employed could display the inhibitory activity (data not shown). The mass spectrum of cholesta-4,6-diene-3-ol used in this study is shown in Figure 9B.

Cholesterol Inhibits naER-E-RAF Dimerization

Sucrose density gradient analysis was carried out to find out whether cholesterol influenced the dimer formation between naER and E-RAF. In the absence of cholesterol, the complex sedimented at 4.8–5.0 S, while in the presence of 20 nM cholesterol, the hormone-binding activity was restricted to the 4.0- to 4.2-S peak, which represented the free naER (Fig. 10A).

Cholesterol Inhibits the Nuclear Binding of naER/E-RAF:

The effect of cholesterol on nuclear binding of the ^{3}H-E_2 naER-E-RAF complex was examined. Cholesterol was observed to inhibit the nuclear binding of ^{3}H-E_2 naER-E-RAF complex in a concentration-dependent manner (Fig. 10B).

Saturation Binding of ^{3}H-Cholesterol to E-RAF

Cholesterol binds with high affinity (kD ~ 1×10^{-10} M) to E-RAF (Fig. 11A,B). The following synthetic derivatives of cholesterol, selected at random, were tested for their capacity to inhibit cholesterol binding to E-RAF. The effect, in terms of their capacity to inhibit cholesterol binding to E-RAF was as follows: (1) cholesteryl stearate (100%); (2) 5α cholestan-3-one (100%); (3) (+) dihydrocholesterol (100%); (4) 5β-cholestan-3-α-ol (85%); (5) cholesta-4,6-dien-3-one (71%); (6) 5-cholesten-3-one (32%); (7) cholesteryl oleate (32%); (8) (+) 4-cholesten-3-one (32%); (9) 5-cholesten-3β-ol-7-one (25%);

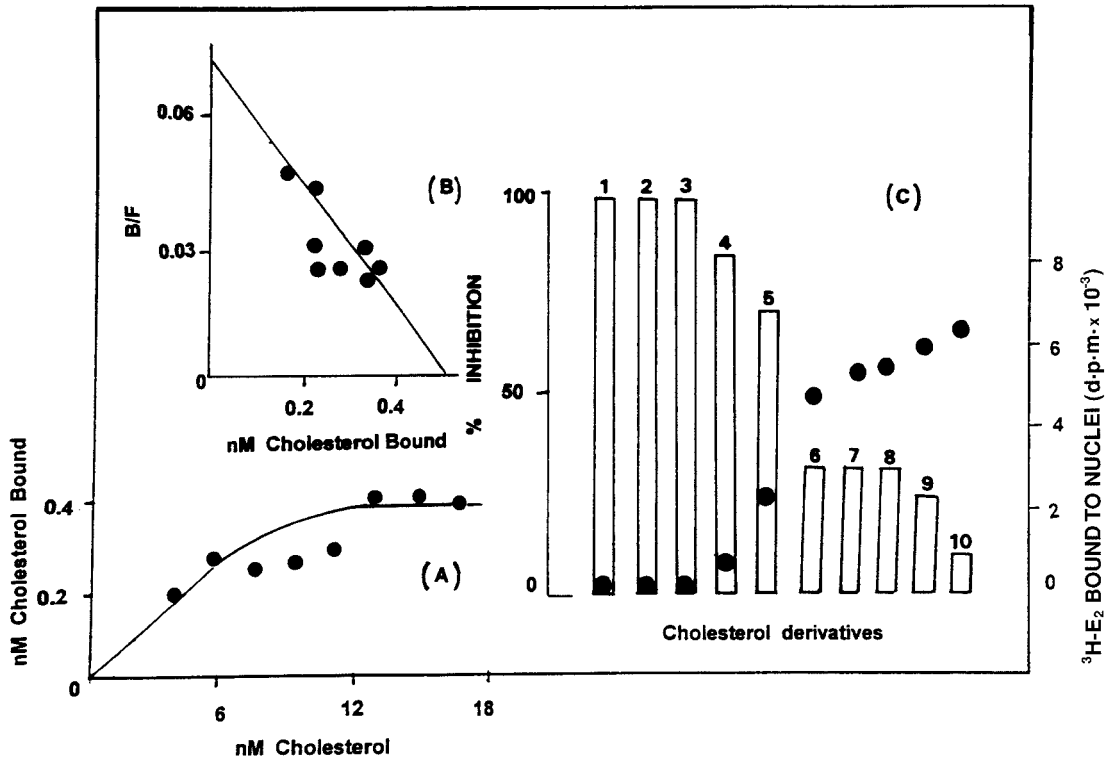

Fig. 11. Binding of ^{3}H-cholesterol to estrogen receptor activation factor (E-RAF). **A:** Saturation binding of ^{3}H-cholesterol to E-RAF was measured using a hydroxyapatite binding assay. **B:** Scatchard plot of the binding data presented in **A**. **C:** Competition studies. Randomly selected derivatives of cholesterol were employed to find out their effectiveness in inhibiting ^{3}H-cholesterol binding to E-RAF and also in preventing the nuclear binding of ^{3}H-E$_2$-nonactivated estrogen receptor (naER)-E-RAF complex. The histograms show the effect (represented as percentage inhibition) of the cholesterol derivatives on ^{3}H-cholesterol binding to E-RAF. The compounds were used at 2 μM concentration, while ^{3}H cholesterol was employed at 20-nm concentrations. The binding was measured using a HAP adsorption assay. The results of the nuclear binding assay are represented by the circles. The nuclei were incubated with ^{3}H-E$_2$-naER and E-RAF in the presence of 20-nm concentrations of the synthetic compounds. The radioactive hormone bound to the nuclei was measured. The synthetic derivatives of cholesterol used were:(1) cholesteryl stearate; (2) 5α cholestan-3-one; (3) (+) dihydrocholesterol; (4) 5β cholestan-3α-ol; (5) cholesta-4,6 diene-3-one; (6) 5 cholesten-3-one; (7) cholesteryl oleate; (8) (+) cholesten-3-one; (9) cholesten-3β-ol-7-one; (10) cholesteryl palmitate.

and (10) cholesteryl palmitate (10%). It was also observed that this property ran parallel to the capacity of the individual compounds to inhibit nuclear binding of the ^{3}H-E$_2$ naER-E-RAF complex (Fig. 11C). The values given in parentheses indicate the percentage inhibition effected by the test compound (2 μM) on the binding of 20 nm ^{3}H cholesterol to E-RAF.

CD Analysis of Cholesterol-E-RAF Interaction

CD measurements were taken to find out whether cholesterol introduced any changes in the tertiary (Fig. 12A) or the secondary (Fig. 12B) structure of the E-RAF. A marginal yet noticeable change was observed in both the cases. However, in the tertiary CD, the change observed at 280 nm was insignificant, apparently indicating that the regions where conformational changes are introduced do not contain tryptophan or tyrosine residues.

Nuclear Entry of FITC-Labeled E-RAF or naER

FITC-labeled naER failed to enter the nucleus on its own (Fig. 13A,F). After dimerization with the E-RAF, the complex remained at the nuclear periphery, incapable of entering the nucleus (Fig. 13B,G). FITC-labeled E-RAF gained an immediate entry into the nucleus (Fig. 13C,H). After dimerization with naER the complex remained at the nuclear periphery (Fig. 13D,I). Exposure of FITC-labeled E-RAF to 20 nM cholesterol prevented the entry of the protein into the nucleus (Fig. 13E,J).

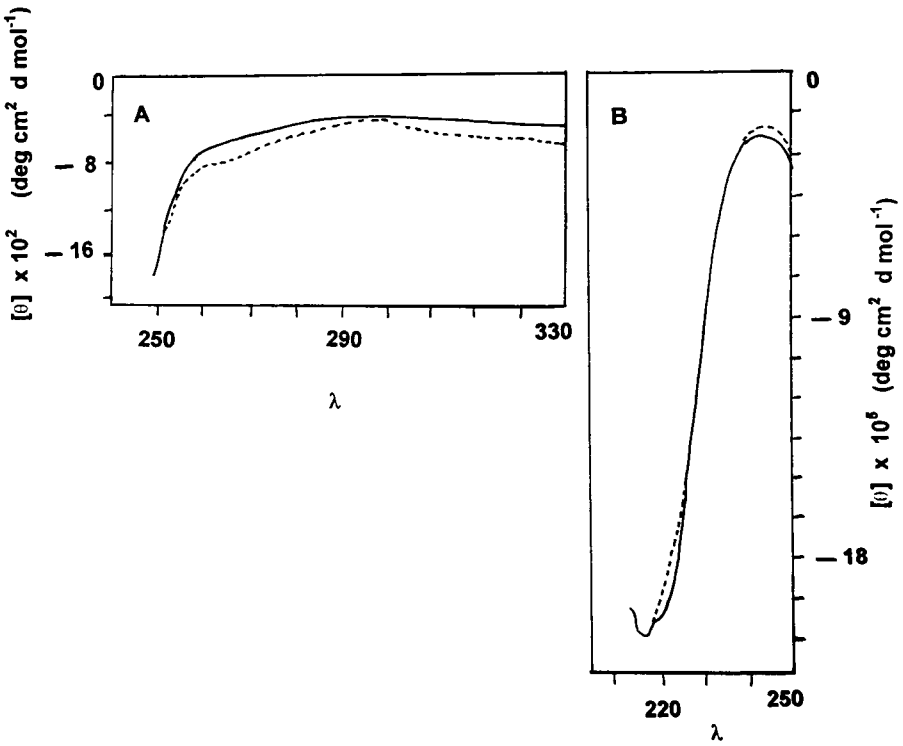

Fig. 12. Circular dichroism (CD) measurement of cholesterol interaction with estrogen receptor activation factor (E-RAF). E-RAF (~3 μg protein per 450 μl) was analyzed in a JASCO spectropolarimeter either in the presence (broken line) or in the absence (solid line) of 20 nM cholesterol. Near-UV CD spectra were taken to detect any change that occurs in the E-RAF conformation either at the tertiary (**A**) or at the secondary (**B**) level.

DISCUSSION

Receptor activation is a molecular mechanism that has been widely recognized by investigators working with different steroid receptor systems [Higgins et al., 1973; Bailly et al., 1980; Grody et al., 1982]. The term refers to the process by which a non-DNA binding steroid receptor acquires the capacity to bind to the DNA. During the early 1970s, it was observed that a heat-stable and dialyzable component endogenous to the mammalian uterus prevented the estrogen receptor-activation process [Sato et al., 1978, 1980]. Cholesterol, as it appears from the data presented in this manuscript, comes close to being identified as the inhibitor described originally by Sato and coworkers.

The estrogen receptor activation process has been studied in our laboratory, in a totally different perspective. The ER under study is not the well-recognized receptor that binds as a homodimer to the estrogen responsive element in the target gene. This alternative ER of our interest, the naER, is not a DNA binding protein. It gains access to the DNA only after it dimerizes with the E-RAF, a DNA-binding protein whose binding domain on the estrogen responsive gene has not yet been identified. Therefore, the "receptor activation" in our studies is the dimerization between the naER and the E-RAF, which, as it appears in the present study, takes place only within the nucleus.

E-RAF exists in two functional forms: I and II [Thampan, 1987]. While E-RAF II destabilizes the DNA double helix, E-RAF I stabilizes it. Consequently, E-RAF II enhances transcription in a reconstituted in vitro transcription system while E-RAF I inhibits it [Thampan, 1989]. Therefore, it will not be out of place to suggest that one functional role of E-RAF may be that of a transcription factor, capable of enhancing or blocking gene expression depending on the form of E-RAF that arrives at the transcription site. Prevention of the nuclear entry of E-RAF, under these conditions, could have a qualitative and quantitative effect on the transcriptional process.

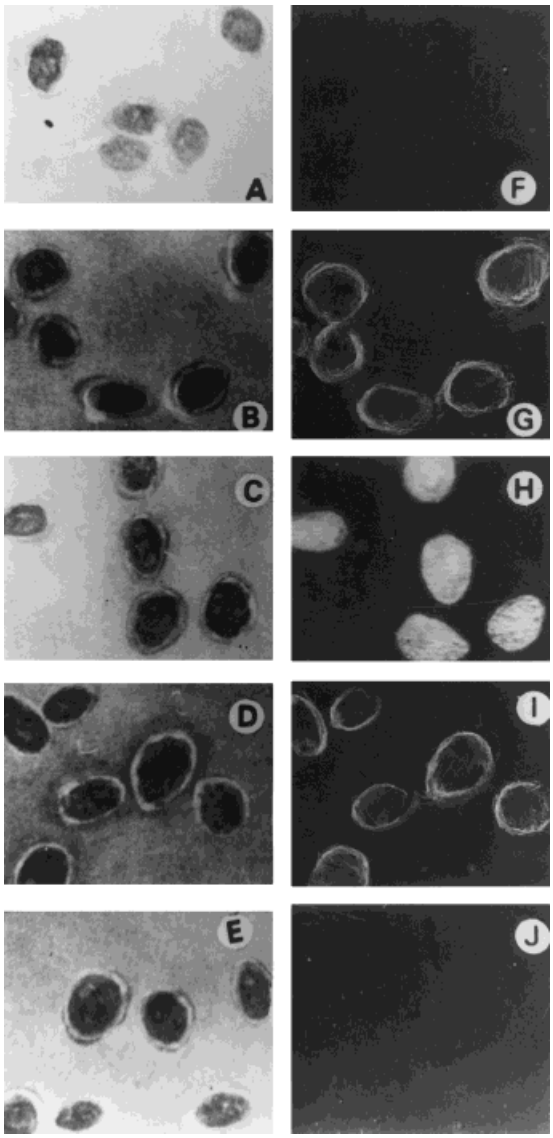

Fig. 13. Nuclear entry of nonactivated estrogen receptor (naER) and estrogen receptor activation factor (E-RAF). Both naER and E-RAF were labeled with FITC and incubated with isolated uterine nuclei under appropriate conditions, as described under Materials and Methods. **A,B,C,D,E:** Phase-contrast micrographs of nuclei, the fluorescence micrographs of which are presented in **F,G,H,I,J**, respectively. **A,F:** Isolated nuclei incubated with FITC-labeled naER alone. **B,G:** Isolated nuclei incubated with FITC-labeled naER and unlabeled E-RAF. **C,H:** Nuclei incubated with FITC-labeled E-RAF alone. **D,I:** Nuclei incubated with FITC-labeled E-RAF and unlabeled naER E,J: Nuclei incubated with FITC-labeled E-RAF and 20 nM cholesterol.

Treatment of postmenopausal women with estradiol reduces the coronary heart diseases risk [Rosenberg et al., 1976] while opening up the risk of cancer of the reproductive tissues [Sherman et al., 1983]. The cholesterol that is under focus is the extracellular and circulating cholesterol. There have been reports earlier pertaining to the estrogen receptor-mediated regulation of cholesterol biosynthesis in breast cancer cell lines [Cypriani et al., 1988]. These results indicate a role for estradiol in cholesterol homeostasis. Whether the low-density lipoprotein (LDL) receptor gene in the uterus is under the regulatory influence of E-RAF, either alone or in combination with the naER, remains to be determined. If this is the case, it will not be difficult to underline the physiological significance of the observation presented in this article. As of now there is no direct evidence available to confirm that the LDL receptor gene is under the influence of the regular ER.

There is an apparent similarity between E-RAF and the sterol-responsive element binding protein (SREBP) reported by the research team of Goldstein and Brown [Hua et al., 1995, 1996]. When the intracellular cholesterol level is low, the SREBP moves out of the endoplasmic reticulum and enters the nucleus. This response is not seen when the intracellular cholesterol level is high. Within the nucleus it binds to the SRE of LDL receptor gene, influencing its transcription. It remains to be seen whether the E-RAF binds to the SRE. If it does, it will be possible to explain the basis for estrogenic regulation of LDL receptor gene expression. Recent experiments carried out in our laboratory indicate that the primary intracellular site where E-RAF is localized is the endoplasmic reticulum (A.P. Govind and R.V. Thampan, manuscript in preparation). These observations indicate that E-RAF remains firmly anchored to a protein of the endoplasmic reticulum in the presence of physiological concentrations of estradiol. As the estradiol concentration decreases, the interaction between the two proteins weakens, resulting in the dissociation of the E-RAF from the complex. This E-RAF will now be free to enter the nucleus, as the studies presented in this report show that E-RAF entry into the nucleus is not mediated by any cytosolic protein.

If the naER-E-RAF heterodimer is a universal mediator of estrogen-regulated gene expression, one would anticipate that prevention of the nuclear entry of E-RAF will inhibit estrogen action completely. There is practically no information available in the literature that would indicate an “anti-estrogenic” action of

cholesterol. The total recovery of classical ER and naER from a given mass of tissue follows a 10:1 ratio. It may signify that at a given stage in estrogen action the average molecular responses influenced by ER and naER may reflect a 10:1 pattern. The naER-E-RAF complex may therefore be responsible for the regulation of an extremely small number of genes that are not influenced by the classical ER and associated proteins. Genes involved in the regulation of cholesterol homeostasis may come under this category. Under such circumstances, cholesterol-dependent blockade of the nuclear entry of E-RAF should lead to biochemical changes associated with cholesterol homeostasis, and not to general physiological changes that may result from the blockade of the regular ER-mediated gene regulatory mechanisms.

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