Biochemistry

 Volume 22, Number 25 December 6, 1983

Accelerated Publications

Effects of 3β-[2-(Diethylamino)ethoxy]androst-5-en-17-one on the Synthesis of Cholesterol and Ubiquinone in Rat Intestinal Epithelial Cell Cultures†

Russell C. Sexton, Sankhavaram R. Panini, Florence Azran, and Harry Rudney*

ABSTRACT: The relationship between cholesterol and ubiquinone synthesis in rat intestinal epithelial cell cultures was examined by using 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one hydrochloride (U18666A). Addition of U18666A to cells caused a greater than 90% inhibition of incorporation of [^{3}H]acetate into cholesterol and an apparent large increase in the incorporation of [^{3}H]acetate and [^{3}H]mevalonate into ubiquinone. However, the incorporation of 4-hydroxy[U-^{14}C]benzoate, a ring precursor of ubiquinone, was unchanged. The apparent increase of ^{3}H incorporation into ubiquinone was found to be due to the formation of a contaminant that has been identified as squalene 2,3:22,23-dioxide. Following incubation of cells with U18666A, its removal from the medium resulted in a decrease in squalene 2,3:22,23-dioxide labeling and a corresponding increase in the polar sterol fraction. These results demonstrate that U18666A inhibits the reaction catalyzed by 2,3-oxidosqualene cyclase (EC 5.4.99.7). As a result, the isoprenoid precursors are diverted not to ubiquinone as has been suggested but to squalene 2,3:22,23-dioxide, a metabolite not on the cholesterol biosynthetic pathway. Removal of the drug allows cyclization of squalene 2,3:22,23-dioxide, leading to formation of compounds with chromatographic properties of polar sterols.

The biosynthetic pathways of cholesterol, ubiquinone, and dolichol share several common steps preceding the branch point at farnesyl pyrophosphate (Rudney, 1970). Studies on the flux of isoprenoid precursors through these pathways have led to the hypothesis that when cholesterol synthesis is blocked beyond the branch point, the precursors are diverted into the other pathways (Faust et al., 1979; Brown & Goldstein, 1980). For example, when rats were fed cholesterol (Krishnaiah et al., 1967; Rao & Olson, 1967) or cells in culture treated with low-density lipoproteins (LDL)[1] (Faust et al., 1979), the incorporation of labeled mevalonate into the side chain of ubiquinone appeared to increase. In each case, however, incorporation of 4-hydroxy[U-^{14}C]benzoate (4-HB) into the ring portion of ubiquinone actually decreased (Ranganathan & Ramasarma, 1975; Nambudiri et al., 1980; Ranganathan et al., 1981). Both cholesterol feeding and LDL treatment lead to substantial lowering of the activity of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.34) and thereby the endogenous production of mevalonate. Nambudiri et al. (1980) therefore postulated that the increased incorporation of trace levels of labeled mevalonate into ubiquinone did not reflect increased synthesis but rather decreased dilution of the tracer by the endogenous mevalonate pool.

In this context, the recent observation (Volpe & Obert, 1982) that the compound 3β-[2-(diethylamino)ethoxy]-androst-5-en-17-one hydrochloride (U18666A) greatly enhanced the incorporation of mevalonate into ubiquinone at the expense of cholesterol in cultured neural cells was of much interest. This compound is reported to decrease cholesterol synthesis by inhibiting desmosterol reductase (Phillips & Avigan, 1963; Bierkamper & Cenedella, 1978; Volpe & Obert, 1982) and also a step preceding lanosterol formation (Cenedella, 1980; Volpe & Obert, 1982). Since U18666A did not appear to affect the activity of HMG-CoA reductase (Volpe

† From the Department of Biological Chemistry, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267. *Received August 29, 1983.* Supported by grants from the National Institutes of Health, NIADDKD (AM-12402), and the National Science Foundation (PCM-7812124). A portion of this work was presented at the annual meeting of the American Society of Biological Chemists, San Francisco, CA, June 1983 (Panini et al., 1983).

[1] Abbreviations: LDL, low-density lipoproteins; 4-HB, 4-hydroxybenzoate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; U18666A, 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one hydrochloride; SO, squalene 2,3-oxide; SDO, squalene 2,3:22,23-dioxide; LPDS, lipoprotein-deficient fetal bovine serum; IEC-6, rat intestinal epithelial cells; TLC, thin-layer chromatography.

0006-2960/83/0422-5687$01.50/0

& Obert, 1982) and, by inference, the intracellular mevalonate pool, the observed stimulation of ubiquinone synthesis strongly supported the precursor diversion hypothesis.

The purpose of the present study was to determine whether the inhibition of cholesterol synthesis by U18666A resulted in true enhancement of ubiquinone synthesis as measured by the incorporation of labeled precursors into both the side-chain and the ring portions of the ubiquinone molecule. In this study, cholesterol and ubiquinone syntheses were measured in an established line of epithelioid cells derived from rat intestinal crypts (IEC-6) (Quaroni et al., 1979; Quaroni & May, 1980). This work extends our observations on regulation of isoprenoid biosynthesis in this cell line, previously shown to regulate cholesterol synthesis in response to cell growth and lipoproteins (Panini et al., 1982). The results herein clearly show that U18666A does not increase ubiquinone synthesis but instead enhances the synthesis of squalene epoxides that may contaminate ubiquinone, depending on the separation procedures used.

Materials and Methods

Chemicals. Sodium [^{3}H]acetate (1.6 Ci/mmol), (*RS*)-[5-^{3}H]mevalonolactone (5.7 Ci/mmol), [4-^{14}C]cholesterol, and L-[U-^{14}C]tyrosine (487 mCi/mmol) were obtained from New England Nuclear (Boston, MA). 4-Hydroxy[U-^{14}C]benzoic acid (360 mCi/mmol) was synthesized by alkali fusion of L-[U-^{14}C]tyrosine by the method of Parson & Rudney (1964). Ubiquinone 9, squalene, lanosterol, and cholesterol were purchased from Sigma (St. Louis, MO). U18666A was a generous gift of Dr. Richard Cenedella, Kirksville College of Osteopathic Medicine. Dulbecco's modified Eagle medium, fetal bovine serum, and other solutions to prepare the growth medium were obtained from Grand Island Biological Co. (Grand Island, NY). Other chemicals used were of analytical grade.

Cell Cultures. Rat intestinal epithelial cells (IEC-6) were obtained initially as a gift from Dr. A. Quaroni, Harvard Medical School, and subsequently from the American Type Culture Collection (CRL-1592). Cells were cultured in monolayers and used between the 9th and 20th passage. Stock cultures were maintained in 10 mL of Dulbecco's modified Eagle medium supplemented with glucose (4.5 g/L), insulin (0.2 unit/mL), penicillin (50 units/mL), streptomycin (50 μg/mL), glutamine (4 mM), and 5% fetal bovine serum in tissue culture dishes (100 × 20 mm) in a humidified incubator with 10% CO_2 atmosphere at 37 °C. Subcultures for experiments were derived from stock cultures by methods previously described (Nambudiri et al., 1980).

Incorporation of Radiolabeled Precursor into Nonsaponifiable Lipids. Cells were seeded on day 0 between 2 and 5 × 10^5 cells in 60 × 15 mm dishes in 3 mL of medium containing 5% fetal bovine serum. On days 1 and 2, the monolayers were refed 2 mL of medium containing 5% (v/v) lipoprotein-deficient serum (LPDS). The LPDS was prepared from fetal bovine serum by ultracentrifugation using potassium bromide for density adjustment (Redding & Steinberg, 1960). On day 3, cells were pretreated with the indicated amounts of the drug U18666A dissolved in 20 μL of 75% (v/v) ethanol. Control dishes received an equal volume of 75% ethanol. One to two hours after drug pretreatment, groups of dishes received radiolabeled compounds and were incubated for the indicated times. After the incubation, the medium was removed, and the dishes were rinsed twice with 2 mL of 0.9% NaCl. The cells were then digested in 1 mL of 0.1 N NaOH for 30 min. An aliquot was removed for protein determination (Schacterle & Pollack, 1973), and the digest was transferred to a glass tube. The culture dish was rinsed with 1 mL of water. To the pooled digest were added pyrogallol (100 mg), carrier amounts of cholesterol (250 μg), lanosterol (100 μg), ubiquinone (250 μg), and squalene (10 μg), and [4-^{14}C]cholesterol (15 000 cpm/tube) as an internal standard. The digest was evaporated to near dryness under N_2. Five milliliters of 1 N KOH in methanol/benzene (4:1) was added and the mixture saponified at 80 °C for 30 min. The saponified mixture was diluted with 3 mL of water and extracted with 20 mL of hexane. The hexane layer was washed twice with 2 mL of water and once with 2 mL of 70% (v/v) ethanol and evaporated under N_2.

TLC Systems I and II. The nonsaponifiable lipids were separated on TLC plates by either of two systems: system I, a one-dimensional TLC separation on 5 × 20 cm glass-backed silica gel 60 plates (Merck 5762) with petroleum ether/acetone (90:10) as the solvent; system II, a two-dimensional TLC separation on 20 × 20 cm glass-backed silica gel 60 plates (Merck 5763). For the first dimension, the lipids were separated as in system I. For the second dimension, a reverse-phase separation, the plates were impregnated with paraffin by dipping them into a solution of 5% (v/v) paraffin oil (Saybolt viscosity 125/135, Fisher Scientific Co., Fair Lawn, NJ) in petroleum ether and then developed in the second dimension with acetone/paraffin-saturated water (85:15) as the solvent.

Radioactivity Incorporation Measurements. The qualitative distribution of radioactivity into the various lipid fractions on the thin-layer chromatograms was determined by scanning the TLC plates in a Packard 7201 radiochromatogram scanner. A radioactive marker applied to one edge of the plates facilitated alignment of radioscans with the chromatograms. Areas on the TLC plates corresponding to the internal lipid standards were visualized by iodine or anisaldehyde (Dunphy et al., 1967) staining. The radioactive peaks were marked, scraped into vials containing scintillation fluid (NEN 950A), and counted in a Beckman LS 3145P liquid scintillation counter. The results are expressed as the mean of duplicate or triplicate determinations. The variation among the samples within a given experiment was less than 10% of the mean.

Preparation of Squalene 2,3-Oxide and Squalene 2,3:22,23-Dioxide. Squalene 2,3-oxide (SO) was prepared by the method of Willet et al. (1967) and its purity checked by thin-layer chromatography using TLC system I. Squalene 2,3:22,23-dioxide (SDO) was prepared by a modification of the method of Field & Holmlund (1977). The dioxide was separated from the crude epoxide mixture by thin-layer chromatography using TLC system I. SDO was eluted from the TLC plate with methanol and then stored under N_2 at −70 °C. The structures of SO and SDO were confirmed by nuclear magnetic resonance and infrared spectra, which agreed with those reported by Willet et al. (1967) for SO and by Field & Holmlund (1977) for SDO. Thin-layer chromatographic characteristics of authentic SO and SDO were determined in five different one-dimensional systems on silica gel 60 plates. The five systems were (1) petroleum ether/acetone (90:10), (2) hexane/ethyl acetate (85:15), (3) hexane/ethyl acetate (70:30), (4) benzene/ethyl acetate (99.5:0.5), and (5) acetone/paraffin-saturated water (85:15) using paraffin-impregnated plates. The respective R_f values for SO and SDO were (1) 0.75, 0.51, (2) 0.62, 0.36, (3) 0.88, 0.76, (4) 0.46, 0.16, and (5) 0.21, 0.56.

Results and Discussion

The effects of U18666A on the incorporation of labeled precursors into cholesterol and ubiquinone of IEC-6 cells are

Table I: Effect of U18666A on Incorporation of Radiolabeled Precursors into Cholesterol and Ubiquinone[a]

radiolabeled precursor and treatment	radioactivity incorpn [dpm (mg of protein)$^{-1}$ h^{-1}] into	
	cholesterol	ubiquinone
[^{3}H]acetate		
control	66 000	300
U18666A (10 ng/mL)	41 500	1 300
U18666A (500 ng/mL)	6 300	79 100
[^{3}H]mevalonolactone		
control	18 400	600
U18666A (10 ng/mL)	14 000	3 000
U18666A (500 ng/mL)	1 600	9 700
4-hydroxy[^{14}C]benzoate		
control		90
U18666A (10 ng/mL)		80
U18666A (500 ng/mL)		90

[a] Cells were grown for 2 days as described under Materials and Methods. On day 3, duplicate dishes received the indicated amounts of U18666A or the solvent. Three hours later, groups of dishes received either [^{3}H]acetate (10 μCi/mL) and 4-hydroxy[U-^{14}C]benzoate (33 nCi/mL) or [5-^{3}H]mevalonolactone (6.6 μCi/mL). Three hours after the addition of the radiolabel, cells were harvested, and the incorporation of radioactivity into the lipids was determined by TLC system I as described under Materials and Methods.

shown in Table I. Since treatment of cells with U18666A (500 ng/mL) decreased incorporation of both acetate and mevalonate into cholesterol to a similar extent, the inhibition of cholesterol synthesis by U18666A appeared to be at a site(s) beyond the formation of mevalonate. Table I also shows that U18666A increased the incorporation of acetate and mevalonate into the polyisoprenoid side chain of ubiquinone but, at the same time, had no effect on ubiquinone ring synthesis from 4-HB. This difference in the incorporation of side-chain and ring precursors into ubiquinone raised the question whether acetate and mevalonate were truly incorporated into the side-chain of ubiquinone or rather into some isoprenoid intermediate with a mobility similar to that of ubiquinone in the TLC system employed.

To answer this question, cells were incubated in the presence and absence of U18666A, and the labeled nonsaponifiable lipids were separated by using TLC system I as described under Materials and Methods. As shown in Figure 1A, in the absence of U18666A, the major radioactive peak corresponded to cholesterol, whereas, in the presence of U18666A, incorporation of radioactivity into cholesterol was substantially decreased. Additionally, there appeared two new labeled compounds, designated H and I, which migrated between ubiquinone and squalene. Neither H nor I was produced in sufficient quantity to be visualized by iodine or anisaldehyde staining. As shown in this typical chromatogram with TLC system I, compound H was not well separated from ubiquinone; thus, a minor contamination from compound H could cause a significant increase in the radioactivity associated with the ubiquinone fraction. In order to achieve a better separation of compound H from ubiquinone, a two-dimensional TLC system (system II) was employed, and the results are presented in Figure 1B. With this system, compounds H and I could be clearly separated from each other and the rest of the lipids. These results clearly demonstrated that estimation of ubiquinone synthesis, as measured by the incorporation of labeled acetate or mevalonate, could be subject to error due to the contamination of the ubiquinone fraction with compound H when a one-dimensional TLC system was employed. Consequently, in order to determine if U18666A increased the incorporation of side-chain precursors into ubiquinone, the

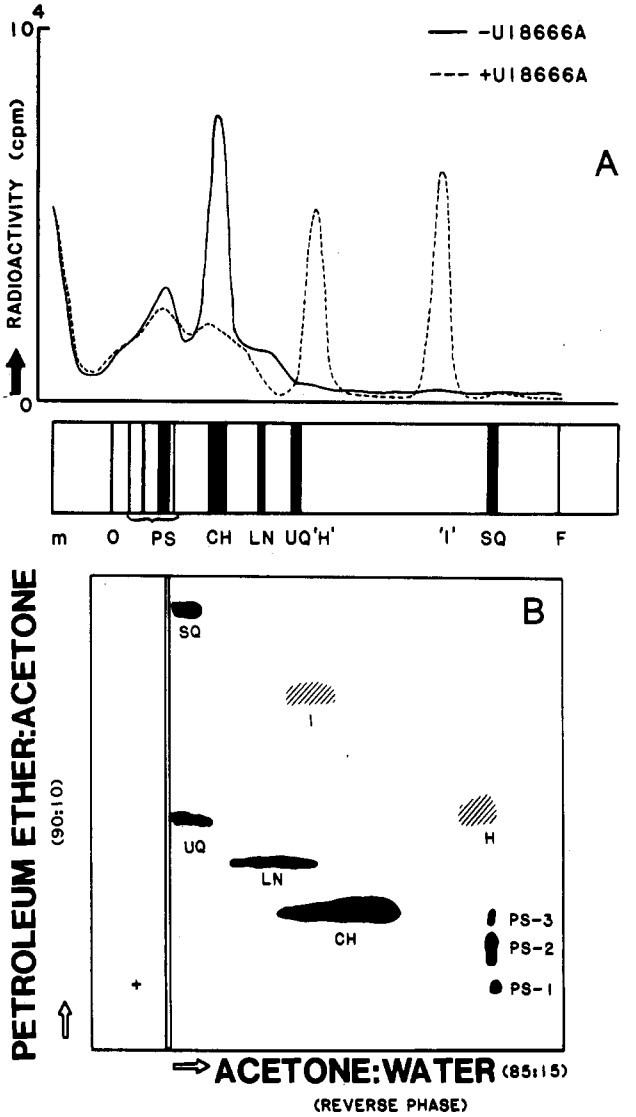

FIGURE 1: (A) Incorporation of [^{3}H]acetate into nonsaponifiable lipids synthesized in the presence and absence of U18666A. IEC-6 cells were grown for 2 days as described under Materials and Methods. On day 3, duplicate dishes were pretreated with U18666A (500 ng/mL) for 2 h, followed by a pulse of [^{3}H]acetate (10 μCi/mL) for 1 h. The nonsaponifiable lipids were separated by TLC system I, and the distribution of radioactivity was determined in a Packard 7201 radiochromatogram scanner as described under Materials and Methods. Abbreviations: m = marker; O = origin; PS = polar sterols; CH = cholesterol; LN = lanosterol; UQ = ubiquinone; SQ = squalene; F = solvent front; H and I = unknown. (B) Separation of compounds H and I from other nonsaponifiable lipids synthesized from [^{3}H]acetate in the presence and absence of U18666A. Conditions and designations for the various lipids were the same as in (A) except that the nonsaponifiable lipids were separated by TLC system II as described under Materials and Methods. Locations of spots H and I were established by measurement of radioactivity.

two-dimensional/partial reverse-phase TLC system was utilized.

The results of using TLC system II to separate the nonsaponifiable lipids after preincubation of IEC-6 cells with U18666A for 2 h, followed by a pulse of [^{3}H]acetate for 1 h, are shown in Figure 2. With increasing concentrations of U18666A, there was a progressive decrease in [^{3}H]acetate incorporation into cholesterol and lanosterol (methyl sterols) coupled with an increased incorporation into compounds H and I (Figure 2A), as previously noted in Figure 1A. At the same time, however, there was no change in the incorporation of radioactivity into squalene or ubiquinone (Figure 2B). This lack of an effect of U18666A on [^{3}H]acetate labeling of the

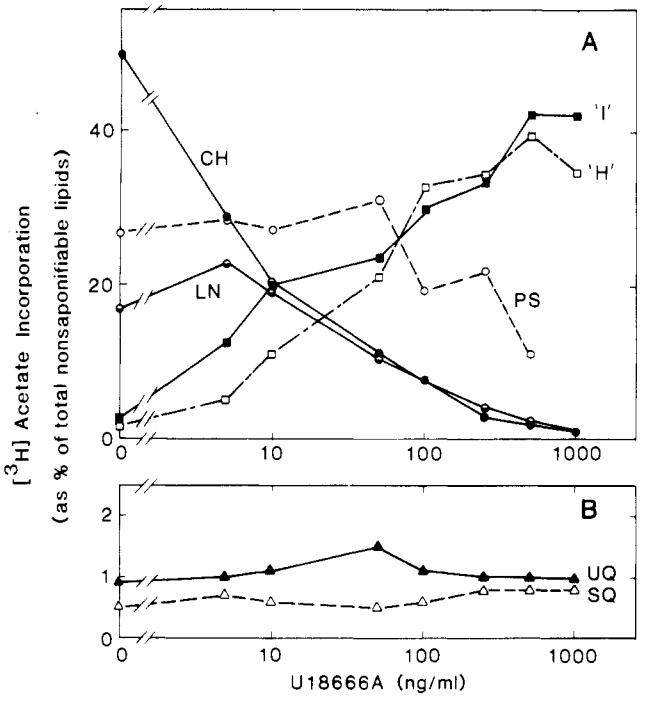

FIGURE 2: Cells were grown for 2 days as described under Materials and Methods. On day 3, triplicate dishes were pretreated with U18666A at the indicated concentrations for 2 h, followed by a pulse of [^{3}H]acetate (10 μCi/mL) for 1 h. The incorporation of radioactivity into the nonsaponifiable lipids was determined after separation by TLC system II. The concentrations of U18666A (ng/mL) and the corresponding values for the incorporation of [^{3}H]acetate into total nonsaponifiable lipids (dpm $\times 10^{-6}$ mg of protein) were as follows: 0, 0.96; 5, 0.67; 10, 0.63; 50, 0.63; 100, 0.94; 250, 1.04; 500, 1.00; 1000, 1.20. Symbol notation in panel A: (●) cholesterol (CH); (⊖) lanosterol (LN); (○) polar sterols (PS); (□) compound H; (■) compound I. Symbol notation in panel B: (▲) ubiquinone (UQ); (△) squalene (SQ).

polyisoprenoid side chain of ubiquinone agreed well with the results of incorporation of 4-HB into the ring portion of ubiquinone shown in Table I. These observations prove that U18666A does not increase ubiquinone synthesis in IEC-6 cells under the conditions employed.

In rat brain tissue, the metabolic site at which U18666A inhibits sterol synthesis was suggested by Cenedella (1980) to be prior to the formation of lanosterol. Volpe & Obert (1982) suggested the inhibition occurred at the squalene synthetase step. Data in Figure 2 also show that treatment of IEC-6 cell cultures with increasing concentrations of U18666A progressively decreased the incorporation of [^{3}H]-acetate into lanosterol while that into squalene was not significantly affected. These results suggested that U18666A inhibited the conversion of squalene to lanosterol. In a separate experiment (data not shown), it was observed that compound H could be effectively labeled by [^{3}H]squalene and that treatment with U18666A (500 ng/mL) enhanced such labeling 5-fold. This indicated that compound H was a biological product of squalene. Inhibition of the enzyme oxidosqualene cyclase (EC 5.4.99.7) has been shown to prevent lanosterol synthesis from squalene and pre-squalene precursors and to lead to the accumulation of squalene epoxides, e.g., squalene 2,3-oxide (SO) and squalene 2,3:22,23-dioxide (SDO) (Corey & Gross, 1967; Atkin et al., 1972; Chang et al., 1979). Field & Holmlund (1977) reported that the dimethyl analogue of U18666A inhibited oxidosqualene cyclase, resulting in accumulation of SO and SDO in yeast and rat liver. As shown in Figure 2, the incorporation of radioactivity into compounds H and I was greatly increased in the presence of U18666A. The possibility that compounds H and I might be the epoxides of squalene was therefore tested.

Compounds H and I were isolated by TLC system II from the nonsaponifiable lipid fraction of IEC-6 cells treated with U18666A and [^{3}H]acetate as described in the legend to Figure 2. Both labeled compounds were spotted separately on five different thin-layer silica plates. Authentic SO and SDO were also spotted as reference standards. Each silica plate was developed in one of five different solvent systems as described under Materials and Methods. On each silica plate, compound H migrated with the same mobility as SDO and compound I migrated with the same mobility as SO. The R_f values of authentic SDO and SO are given under Materials and Methods. The identities of compounds H and I were further confirmed by acid hydrolysis of mixtures of H with authentic SDO and I with authentic SO by the method of Willett et al. (1967). Extracts of the hydrolyzed mixtures, containing greater than 90% of the original radioactivity in compounds H and I, were subjected to one-dimensional TLC analysis on silica plates using hexane/ethyl acetate (85:15) as the developing solvent. All of the radioactivity of compounds H and I recovered off each chromatogram was found to comigrate with the corresponding products of the acid hydrolysis of authentic SDO and SO. This firmly established the identity of compound H as squalene 2,3:22,23-dioxide and compound I as squalene 2,3-oxide.

As previously noted, Volpe & Obert (1982) reported enhanced ubiquinone synthesis in C-6 glial cells treated with U18666A. These authors separated the nonsaponifiable lipids on a one-dimensional TLC system with chloroform as the developing solvent. The results presented in this paper demonstrate that the apparent enhancement of ubiquinone synthesis by U18666A (Table I) was due to an incomplete separation of ubiquinone (R_f 0.45) from highly labeled SDO (R_f 0.51) when our TLC system I was employed. When chloroform was used to separate the nonsaponifiable lipids, ubiquinone was well resolved from SDO (R_f 0.48) but comigrated with SO (R_f 0.75), the other highly labeled epoxide of squalene. Thus, the artifactual enhancement of ubiquinone synthesis by U18666A could be observed with either TLC system.

The data in Figure 2 also suggest that incubation of IEC-6 cells with U18666A may inhibit the incorporation of [^{3}H]-acetate into compounds more polar than cholesterol. However, the extent of such inhibition varied from experiment to experiment. Such polar compounds as 24,25-oxidolanosterol (Corey & Gross, 1967; Shishibori et al., 1973; Field & Holmlund, 1977) and 24(*S*),25-epoxycholesterol (Nelson et al., 1981a,b) have been identified as enzymatic products of SDO in rat liver. In view of the hypothesis that oxygenated sterol compounds may be part of a natural mechanism regulating cholesterol biosynthesis (Kandutsch et al., 1978; Gibbons et al., 1980; Imai et al., 1980), the ability of IEC-6 cells to metabolize SDO to similar products was examined. IEC-6 cells were first incubated in medium containing U18666A (500 ng/mL) and [^{3}H]acetate for 24 h to maximize the accumulation of SDO and then in medium without U18666A or labeled acetate for another 5 h. The distribution of radioactivity into the various nonsaponifiable lipids using TLC system II for lipid separation is shown in Table II. In the control cells without U18666A in the medium, 64% of the incorporated radioactivity into nonsaponifiable lipid was associated with cholesterol, 33% with compound(s) more polar than cholesterol and approximately 1% with the combined squalene epoxides. In cells preincubated with U18666A, the incorporation of

Table II: Conversion of Squalene 2,3:22,23-Dioxide to More Polar Compounds upon Removal of U18666A from the Medium[a]

	[^{3}H]acetate incorpn (% of total nonsaponifiable lipids)		
		U18666A (500 ng/mL)	
nonsaponifiable lipids	control	present for 24 h	5 h after removal
polar compounds	32.6	28.7	52.1
cholesterol	64.2	1.7	2.2
lanosterol	1.8	0.2	0.8
ubiquinone	0.2	0.2	0.1
squalene 2,3:22,23-dioxide	0.6	62.0	38.0
squalene 2,3-oxide	0.5	7.1	6.1
squalene	0.1	0.1	0.1

[a] Cells were grown for 2 days as described under Materials and Methods. On day 3, duplicate dishes were pretreated with U18666A (500 ng/mL) for 2 h, followed by the addition of [^{3}H]acetate (10 μCi/mL). After 24 h of incubation, the control dishes and one set of U18666A-treated dishes were harvested. The remaining U18666A-treated dishes were washed with 0.9% NaCl and refed fresh medium without U18666A or labeled acetate. The refed cells were incubated an additional 5 h before harvesting. Incorporation of radioactivity into the various nonsaponifiable lipids was determined by TLC system II as described under Materials and Methods. The values for 100% incorporation were 0.8×10^6 dpm/mg of protein (control), 1.1×10^6 dpm/mg of protein (U18666A treated), and 1.1×10^6 dpm/mg of protein (U18666A removed).

radioactivity into cholesterol was inhibited by 97%. Concomitantly, the incorporation into both SO and SDO increased such that nearly 70% of the total radioactivity was found in these two squalene epoxide fractions. When cells were incubated for an additional 5 h in fresh medium without U18666A and radiolabeled acetate, the accumulated radioactivity in SDO decreased from 62% to 38% of the total. This decrease in radioactivity associated with SDO was matched by an equivalent increase in radioactivity associated with the polar compounds (28% to 52% of total). Such redistribution of radioactivity following the removal of the drug was observed consistently with both IEC-6 cells and a human skin fibroblast cell line (GM-43) (data not shown). This indicates that cellular metabolism of SDO resulted in the formation of polar products, in agreement with earlier observations (Field & Holmlund, 1977; Chang et al., 1979).

The identities of the polar compounds formed from SDO and their possible role in the regulation of cholesterol biosynthesis are currently under investigation. Preliminary data (S. R. Panini, R. C. Sexton, and H. Rudney, unpublished results) have shown that incubation of IEC-6 cells with SDO resulted in an inhibition of HMG-CoA reductase, the major rate-limiting enzyme in the sterol biosynthetic pathway. This inhibition could be prevented by pretreating the cells with U18666A, suggesting that the cyclization of SDO to polar sterols may be a prerequisite for the inhibition of HMG-CoA reductase. These results lend further support to the original hypothesis of Kandutsch et al. (1978) that oxygenated derivatives of lanosterol and cholesterol may arise endogenously and influence the biosynthesis of cholesterol. Our data show that oxygenated sterol-like compounds could also be formed by a diversion of squalene to SDO when the enzyme oxidosqualene cyclase is partially inhibited. The potential significance of this enzyme in the regulation of cholesterol biosynthesis is therefore suggested, and further investigation is under way.

In view of our observation that ubiquinone could be contaminated by squalene epoxides during routine TLC separation of neutral lipids, the possibility is raised that such contamination may also account for earlier reports of increased mevalonate incorporation into ubiquinone upon treatment of cells in culture with LDL (Faust et al., 1979; Nambudiri et al., 1980). Future studies utilizing the two-dimensional TLC system described in this paper will explore the relative roles of precursor diversion (Faust et al., 1979), of changes in mevalonate pool size (Nambudiri et al., 1980), and of contaminants (this paper) in the observed increase in ubiquinone synthesis.

References

Atkin, S. D., Morgan, B., Baggaley, K. H., & Green, J. (1972) *Biochem. J. 130*, 153–157.

Bierkamper, G. G., & Cenedella, R. J. (1978) *Brain Res. 150*, 343–351.

Brown, M. S., & Goldstein, J. L. (1980) *J. Lipid Res. 21*, 505–517.

Cenedella, R. J. (1980) *Biochem. Pharmacol. 29*, 2751–2754.

Chang, T. Y., Schiavoni, E. S., McCrae, K. R., Nelson, J. A., & Spencer, T. A. (1979) *J. Biol. Chem. 254*, 11258–11263.

Corey, E. J., & Gross, S. K. (1967) *J. Am. Chem. Soc. 89*, 4561–4562.

Dunphy, P. J., Kerr, J. D., Pennock, J. F., & Whittle, K. J. (1967) *Biochim. Biophys. Acta 136*, 136–147.

Faust, J. R., Goldstein, J. L., & Brown, M. S. (1979) *Arch. Biochem. Biophys. 192*, 86–99.

Field, R. B., & Holmlund, C. E. (1977) *Arch. Biochem. Biophys. 180*, 465–471.

Gibbons, G. F., Pullinger, C. R., Chen, H. W., Cavenee, W. K., & Kandutsch, A. A. (1980) *J. Biol. Chem. 255*, 395–400.

Imai, H., Werthessen, W. T., Subramanyam, V., LeQuesne, P. W., Soloway, A. H., & Kanisawa, M. (1980) *Science (Washington, D.C.) 207*, 651–653.

Kandutsch, A. A., Chen, H. W., & Heiniger, H. G. (1978) *Science (Washington, D.C.) 201*, 498–501.

Krishnaiah, K. V., Joshi, V. C., & Ramasarma, T. (1967) *Arch. Biochem. Biophys. 121*, 147–153.

Nambudiri, A. M. D., Ranganathan, S., & Rudney, H. (1980) *J. Biol. Chem. 255*, 5894–5899.

Nelson, J. A., Steckbeck, S. R., & Spencer, T. A. (1981a) *J. Biol. Chem. 256*, 1067–1068.

Nelson, J. A., Steckbeck, S. R., & Spencer, T. A. (1981b) *J. Am. Chem. Soc. 103*, 6974–6975.

Panini, S. R., Sexton, R. C., & Rudney, H. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1215.

Panini, S. R., Sexton, R. C., & Rudney, H. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1836.

Parson, W. W., & Rudney, H. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51*, 444–450.

Phillips, W. A., & Avigan, J. (1963) *Proc. Soc. Exp. Biol. Med. 112*, 233–236.

Quaroni, A., & May, R. J. (1980) *Methods Cell Biol. 21*, 403–427.

Quaroni, A., Wands, J., Trelstad, R. L., & Isselbacher, K. J. (1979) *J. Cell Biol. 80*, 248–265.

Radding, C. M., & Steinberg, D. (1960) *J. Clin. Invest. 39*, 1560–1569.

Ranganathan, S., & Ramasarma, T. (1975) *Biochem. J. 148*, 35–39.

Ranganathan, S., Nambudiri, A. M. D., & Rudney, H. (1981) *Arch. Biochem. Biophys. 210*, 592–597.

Rao, K. S., & Olson, R. E. (1967) *Biochem. Biophys. Res. Commun. 26*, 668–673.

Rudney, H. (1970) *Biochem. Soc. Symp. No. 29*, 89-103.
Schacterle, G. R., & Pollack, R. L. (1973) *Anal. Biochem. 51*, 654-655.
Shishibori, T., Fukui, T., & Suga, T. (1973) *Chem. Lett.* 1289-1292.
Volpe, J. J., & Obert, K. A. (1982) *J. Neurochem. 38*, 931-938.
Willett, J. D., Sharpless, K. B., Lord, K. E., Van Tamelen, E. E., & Clayton, R. B. (1967) *J. Biol. Chem. 242*, 4182-4191.

Articles

Purification and Properties of a Type β Transforming Growth Factor from Bovine Kidney†

Anita B. Roberts,* Mario A. Anzano, Chester A. Meyers, Janusz Wideman, Russell Blacher, Yu-Ching E. Pan, Stanley Stein, S. Russ Lehrman, Joseph M. Smith, Lois C. Lamb, and Michael B. Sporn

ABSTRACT: Type β transforming growth factor (TGF-β) has been purified 200000-fold from bovine kidneys. This peptide is characterized by its ability to induce anchorage-dependent normal rat kidney cells to grow in soft agar in the presence of epidermal growth factor (EGF); TGF-β is not mitogenic for cells grown in monolayer culture. Purified TGF-β does not compete with EGF for binding to membrane receptors. The concentration of TGF-β required to elicit a half-maximal response for formation of colonies >3100 μm^2 in the soft agar assay is 2-3 pM (55 pg/mL) when assayed in the presence of 0.8 nM EGF (5 ng/mL). The four-step purification procedure which includes chromatography of acid-ethanol tissue extracts on polyacrylamide sizing gels, cation exchange, and two steps of high-pressure liquid chromatography results in a 10% overall yield of colony-forming activity with a recovery of 3-4 μg/kg. Amino acid analysis of purified TGF-β shows 16 half-cystine residues per mole. Analysis of the purified polypeptide by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels indicates that TGF-β is composed of two closely related polypeptide chains cross-linked by disulfide bonds. In the absence of β-mercaptoethanol, the colony-forming activity is associated with a single silver-staining band of molecular weight 25 000; in the presence of β-mercaptoethanol, the TGF-β is converted to an inactive species that migrates as a single band of molecular weight 12 500-13 000. Sequence analysis indicates that at least the first 15 N-terminal amino acids of the two TGF-β subunits are identical.

Two years ago, a new set of acid-stable polypeptide growth factors was described that had the property of acting together with epidermal growth factor (EGF)[1] to induce normal anchorage-dependent indicator cells to grow under anchorage-independent conditions (Roberts et al., 1981, 1982b). The ability to induce anchorage-independent growth had previously been ascribed to crude preparations of sarcoma growth factor by De Larco & Todaro (1978). More recently, it has become clear that these polypeptides all belong to a larger family of transforming growth factors (TGFs) (Roberts et al., 1982a, 1983b) operationally defined by their ability to reversibly induce certain cells to express the transformed phenotype as measured by loss of density-dependent inhibition of growth, overgrowth, and acquisition of both a transformed morphology and the ability to form progressively growing colonies under anchorage-independent conditions (De Larco & Todaro, 1978; Roberts et al., 1981).

Recently, evidence has demonstrated that the transforming activity of these polypeptides cannot be ascribed to any one TGF acting alone on the cell but rather requires the combined action of two TGF subsets which we have designated type α and type β TGFs (Anzano et al., 1982; Roberts et al., 1983b). These two subsets of the TGF family are distinguished by both biological and biochemical properties. Members of the first group, designated TGF-α, are characterized by their ability to compete for binding to the EGF receptor (De Larco & Todaro, 1978, 1980; Todaro et al., 1980). Polypeptides belonging to this class are all single-chain polypeptides of molecular weight 5000-7000, and all contain three intrachain disulfide bonds (Marquardt et al., 1983). Type α TGFs have recently been purified to homogeneity from the conditioned media of both human and rodent transformed cell lines (Marquardt & Todaro, 1982; Twardzik et al., 1982). Since EGF shares extensive amino acid sequence homology with type α TGFs (Marquardt et al., 1983) and can also fully potentiate the soft agar colony-forming response in the presence of type β TGFs (Anzano et al., 1982; Roberts et al., 1982b), it may also be classified as a type α TGF. A second TGF subset, designated TGF-β, does not bind to the EGF receptor (Roberts et al., 1982b). Recent reports from our laboratory and data presented in this paper have demonstrated that type β TGFs from both neoplastic and nonneoplastic sources are of higher molecular weight (25 000) than the type α TGFs and appear to contain disulfide bonds that are necessary for biological

† From the Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205 (A.B.R., M.A.A., C.A.M., S.R.L., J.M.S., L.C.L., and M.B.S.), and the Department of Molecular Genetics, Hoffmann-La Roche, Inc., Nutley, New Jersey 07110 (J.W., R.B., Y.-C.E.P., and S.S.). *Received May 16, 1983.*

[1] Abbreviations: EGF, epidermal growth factor; NRK, normal rat kidney; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; TGF, transforming growth factor; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.