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EFFECT OF A HYPOCHOLESTEROLEMIC AGENT ON CHOLESTERYL ESTER METABOLISM IN GLIOBLASTOMA CELLS

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Abstract—The hypocholesterolemic agent 3β-(2-diethylaminoethoxy)androst-5-en-17-one·HCl (U18666A) is known to induce experimental epilepsy. The possibility that this drug interferes with cholesteryl ester formation in glioblastoma cells was examined. The incorporation of radioactive oleic acid into cellular cholesteryl ester was drastically and specifically inhibited by U18666A. The inhibitory effect of U18666A persisted in different oleic acid concentrations. Kinetic studies revealed the rapidity of U18666A action. U18666A was found to be ineffective in inhibiting acyl-CoA : cholesterol acyltransferase activity when it was added directly to the cell homogenates. In contrast, the acyltransferase activity was greatly diminished in homogenates derived from U18666A-treated cells. Thus, U18666A appeared to block cellular cholesteryl ester biosynthesis by indirectly inactivating acyl-CoA : cholesterol acyltransferase activity in a cell-dependent manner. The potent inhibition of cholesteryl ester formation by U18666A represents one unique aspect of the drug which might contribute to its ability to induce chronic epileptiform activity.

A positive correlation between the content of plasma cholesterol and atherosclerotic diseases has aroused intensive interest in hypocholesterolemic drugs [1]. However, potential clinical applications of some drugs are marred by their serious side effects. U18666A, one of these drugs, impairs the cholesterol biosynthesis and alters the structure and function of the developing brain [2]. Chronic treatment of newborn rats with U18666A induces epilepsy [3]. For this reason, U18666A is not clinically used. However, this represents an excellent experimental system for studying the potential involvement of membrane lipids in epilepsy.

Cholesteryl ester is normally absent in the mature brain, but exists in a significant quantity only in the developing brain [4]. The transient existence of cholesteryl ester during the active period of myelination indicates that cholesteryl ester plays an important role in myelinogenesis. Also, since U18666A is capable of inducing epileptiform activity only if it is administered to immature animals [3], it occurred to us that cholesterol esterification could be related to the action of U18666A.

We recently characterized cholesteryl ester formation in glioblastoma cells and found that acyl-CoA : cholesterol acyltransferase (ACAT) was exclusively responsible for the cholesteryl ester synthesis in these cells [5]. This provides us with a solid enzymological foundation for using glioblastoma cells as a model system to investigate U18666A-induced alterations in cholesterol esterification. This is the first report indicating that U18666A specifically blocks glial cholesterol esterification.

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MATERIALS AND METHODS

[^{3}H]Oleic acid and [1-^{14}C]oleoyl-CoA were purchased from the New England Nuclear Corp. (Boston, MA). Cholesterol, squalene, lanosterol, desmosterol, cholesteryl oleate, and oleoyl-CoA were obtained from the Sigma Chemical Co. (St. Louis, MO), glioblastoma C-6 cells from the American Type Tissue Culture Co. (Rockville, MD), and fetal calf serum (FCS) and Dulbecco's modified Eagle medium (DME) from the Grand Island Biological Co. (Grand Island, NY).

The procedures for measuring the incorporation of radioactive oleic acid into intracellular cholesteryl oleate in the intact cell experiment and the enzymic ACAT assay using cell-free preparations were adopted from established procedures [6, 7]. Slight modifications were as follows. Glioblastoma cells were grown in DME containing 10% FCS (heat inactivated). Two million cells were seeded in each 100 × 20 mm culture dish on day 1. On day 2, the medium was changed to DME + 2 mg/ml lipoprotein deficient serum (LPDS) for some of the experiments. On day 4, the cells were ready for experimentation. Unless otherwise stated, the experiments were run in the presence of FCS. The medium was removed from each dish and the cells were washed twice with DME. Medium (4 ml) containing FCS or LPDS was added to each dish along with the various compounds. They were incubated for 3 hr at 37° in a humidity controlled incubator. A 40-μl sample of a solution containing 8×10^6 cpm of [^{3}H]oleic acid in 0.4 μmole oleate-albumin [8] was

added to each dish, and they were returned to the incubator for 2 more hours. The cells were then washed twice with 2 ml of ice-cold phosphate-buffered saline (PBS), and the dishes were frozen immediately.

The cells were processed by scraping each dish with a cell scraper and putting the suspension in a screw cap 13 × 100 mm test tube. The dishes were rinsed twice with ice-cold PBS to ensure the transfer of all the cells. A 600-μl portion from the total of 6 ml was removed for protein determination. The remaining solutions containing 5.4 ml were then centrifuged at 900 *g* for 10 min after which the supernatant fraction was discarded. The pellet was resuspended in 0.1 ml of PBS. A 4-ml sample of chloroform–methanol (2:1, v/v) was added, and the tubes were vortexed. The tubes were centrifuged at 900 *g* for 10 min, and supernatant fraction was then transferred to test tubes containing 0.15 mg of cholesteryl oleate and 20,000 cpm of [^{14}C]cholesteryl oleate which were used to facilitate visualization of the spot on thin-layer chromatography and determine the recovery (about 80%) respectively. After vortexing, 0.9 ml of PBS was added to each tube and they were vortexed again. The layers were then separated by centrifuging for 10 min. A 50-μl sample of each phase was counted in a beta counter to check the radioactivity. An 800-μl portion of the lower phase was removed to a conical centrifuge tube, and the solvent was removed by evaporation in a rotary evaporator. Hexane (60 μl) was added to the sample, and the resulting solution was streaked on a Brinkmann Sil-25 thin-layer chromatography plate. The plates were developed in a tank containing heptane–ether–acetic acid (85:15:2, by vol.). After drying the plates, the spots were visualized in an iodine tank. The spots corresponding to the cholesteryl oleate were scraped off and counted in scintillation vials containing 6 ml of 3a70b scintillation fluid (Research Products International).

The ACAT assay of glial homogenates was done according to Brown *et al.* [7], except for the following modifications. (The characterization of the glial ACAT assay will be classified elsewhere [5].) Glioblastoma cells (2 × 10^6 cells/dish) were seeded on day 1 in 100 × 20 mm culture dishes. On day 2, the medium in the dishes was changed to DME + 2 mg/ml LPDS for some experiments. On day 4, the cells were incubated with various compounds for 3 hr. The cells were washed twice with PBS, scraped off in 6 ml of PBS, and centrifuged at 2000 rpm for 10 min. The pellet was then resuspended in buffer containing 20 mM potassium phosphate (pH 7.4), 2 mM dithiothreitol, and 2 mM EDTA and sonicated for 20 sec using an Artec sonic dismembrator. Membrane (50 μl, 0.25 mg protein) was incubated with a mixture containing 50 mM potassium phosphate (pH 7.4), 2 mM dithiothreitol, 1.2 mg bovine serum albumin (fatty acid free), 5 μg cholesterol (in acetone) and 40 μM [^{14}C]oleoyl coenzyme A (5 × 10^5 cpm/tube) in a volume of 200 μl. This mixture was incubated at 37° for 1 hr after which the reaction was stopped with the addition of 4 ml of chloroform–methanol (2:1, v/v). The reaction for glial ACAT was found to be linear up to 1 hr [5]. Water (800 μl) and cholesterol oleate (0.15 mg) were added, and the tubes were vortexed. After the two layers separated, 800 μl of the bottom layer was removed and dried in a rotary evaporator. Cholesteryl ester determination was similar to that described above.

Cholesteryl ester spots, formed in both types of experiments, were positively identified in the following manner. The radioactive spots, localized by the adjacent standard, were scraped off and eluted with chloroform. After condensation in volume, the samples were analyzed by two additional TLC systems: (i) petroleum ether (b.p. 38–47°)–ethyl ether–acetic acid (80:20:1, by vol.), $R_f = 0.77$, and (ii) cyclohexane–chloroform (5:50, v/v), $R_f = 0.61$. It was found that more than 98% of the radioactivity that was associated with cholesteryl ester in the standard TLC migrated with cholesteryl ester in these two TLC systems.

Each experiment point is the average of three separate dishes. The standard deviations were less than 10% (average about 7%) of the mean values.

RESULTS

Nerve cells in tissue culture offer an excellent system for studying the dynamic aspects of metabolic changes [9, 10]. We used cultured glioblastoma cells to investigate lipid metabolism altered by U18666A.

Glial cholesteryl ester biosynthesis was measured routinely by the incorporation of radioactive oleic acid into cholesteryl ester in cells. The dose–response curve of U18666A in this sytem is illustrated in Fig. 1. Cellular cholesterol ester synthesis was inhibited substantially by U18666A. The concentration of U18666A required to achieve 50% of the maximal effect of the drug was about 150 ng/ml. Interestingly, U18666A could not completely block cholesterol esterification. The residual basal activity, which

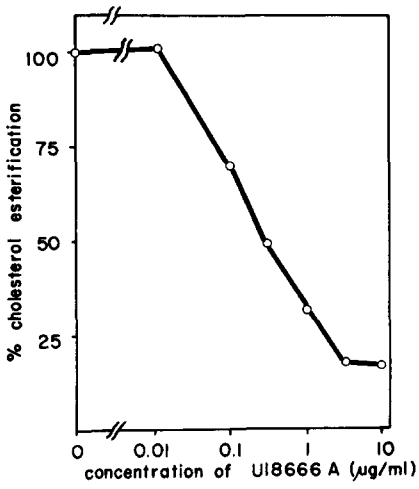

Fig. 1. Concentration curve of U18666A inhibition. Cells were grown in FCS for 3 days. Cells were preincubated with U18666A for 3 hr and then labeled with radioactive oleic acid for 3 hr. The cholesterol esterification capacity of cells in the absence of U18666A was 1190 pmoles · hr^{-1} · (mg protein)$^{-1}$. The cholesteryl ester formed was determined as described in Materials and Methods.

could not be further suppressed by higher concentrations of U18666A, was about 20%. The effect of U18666A appeared to be specific since it did not have any significant effect on the incorporation of radioactive oleic acid into phosphatidylcholine (data not shown).

The effect of incubation time with U18666A was investigated (Fig. 2). The maximum inhibition of cholesterol esterification was achieved with between 2 and 4 hr of preincubation with U18666A. (It was also inhibitory when incubation times were less than 2 hr.) The inhibitory activity of U18666A was evaluated using different oleic acid concentrations (Fig. 3). U18666A was an effective inhibitor in all oleic acid concentrations used.

In an *in vitro* study, it was demonstrated that squalene and desmosterol, intermediates in the cholesterol biosynthetic pathway, accumulate in the presence of U18666A [11]. The possibility exists that these two intermediates may be responsible for the inhibitory effect of U18666A. For this reason, we tested the effects of squalene and desmosterol on cellular cholesterol esterification. It was found that squalene and desmosterol were stimulatory to cholesterol esterification (data not shown). Volpe *et al.* [12] also reported that demosterol stimulates glial cholesterol esterification. The results indicated that the inhibitory action of U18666A was not due to the accumulation of squalene and desmosterol.

We previously established that oxygenated cholesterols such as 25-hydroxycholesterol and 7-ketocholesterol could also enhance cholesterol esterification by increasing ACAT activity in glioblastoma cells [5]. Similar observations were reported by others in nonneuronal cells. Oxygenated cholesterols were proposed to be the true regulators of cholesterol metabolism. The question of whether or not

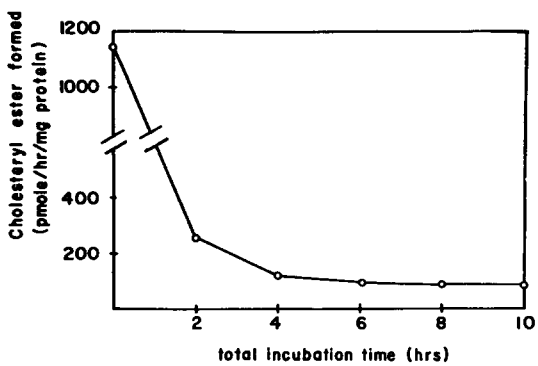

Fig. 2. Rate of incorporation of [^{3}H]oleic acid into cholesteryl ester as a function of time of preincubation with U18666A. Cell monolayers were preincubated in FCS containing U18666A (1 μg/ml) for a certain period of time, after which [^{3}H]oleic acid was added for 2 hr. Zero time represented experiments in which the cells were labeled for 2 hr with oleic acid in the absence of U18666A. The time indicated in the figure included both the time of preincubation with U18666A and time of labeling with oleic acid. The point at 2 hr was obtained from the coincubation for 2 hr with radioactive oleic acid and the drug. The 10-hr point represents experiments in which cells were treated with U18666A for 8 hr before the addition of oleic acid. The time of labeling was 2 hr for all experiments.

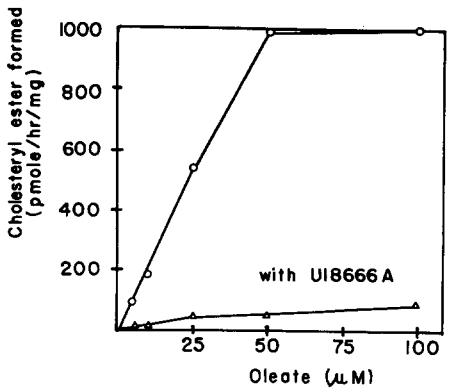

Fig. 3. Rate of incorporation of oleic acid into cholesteryl ester as a function of concentration of oleic acid. Cell monolayers were preincubated in the presence (△) or absence (○) of U18666A (1 μg/ml) for 3 hr. [^{3}H]Oleic acid was then added to dishes for 2 hr. The cells were processed according to the procedures described in Materials and Methods.

U18666A could block the oxygenated cholesterol-induced cholesterol esterification was tested. As can be seen in Table 1, 7-ketocholesterol-enhanced cholesterol esterification was affected by U18666A. Thus, cholesterol esterification, activated by either 7-ketocholesterol or by lipoprotein, was sensitive to U18666A.

The effectiveness of U18666A in inhibiting ACAT activity in homogenates was investigated (Table 2). It was found that the direct addition of U18666A to the assay mixture failed to inhibit ACAT activity. In contrast, a lower ACAT activity was detected in homogenates prepared from U18666A-treated cells. Therefore, cell-dependent inhibition of cellular cholesterol esterification by U18666A is mediated by a permanent change in the ACAT molecule.

DISCUSSION

It has been thought that fatty acids can react directly with cholesterol to form cholesteryl ester in neural tissues [13, 14]. In contrast, ACAT has been found to play an important role in cholesteryl ester biosynthesis in nonneural cells [15–17]. We recently resolved this discrepancy by demonstrating that the CoA thioester of fatty acid is the physiological substrate for cholesteryl ester formation in neural tissues and that ACAT is responsible for cholesteryl ester formation in glioblastoma cells [5]. Taking advantage of this new information, we now report that U18666A is a potent inhibitor of the biosynthesis of cholesteryl ester and of ACAT activity in intact glioblastoma cells.

Since cholesterol, not cholesteryl ester, is associated with membranes, the interconversion of these two forms of cholesterol is expected to regulate the structure and function of the biological membranes. For this reason, U18666A may critically alter the biological activity of the membrane by blocking the conversion of cholesterol to cholesteryl ester. This U18666A activity may pertain to its epileptogenic characteristics.

Table 1. Sensitivity of 7-ketocholesterol-enhanced cholesterol esterification by U18666A*

Compound	Cholesterol esterification	
	pmoles·hr^{-1}·mg^{-1}	%
None	488 ± 12	100
7-Ketocholesterol	1120 ± 33	230
7-Ketocholesterol + U18666A	810 ± 150	165
25-Hydroxycholesterol	1230 ± 17	252

* This experiment was similar to that of Fig. 1, except that cells were grown in LPDS. The final concentrations of U18666A and 7-ketocholesterol were 4 and 10 μg/ml respectively. Values are means ± S.D.

3-Hydroxymethyl-3-glutaryl-CoA reductase was subjected to feedback inhibition by cellular cholesterol. However, the inhibition was only partial. Complete inhibition could only be achieved by supplementing the medium with mevalonate. This observation was explained by a proposal that sterol and nonsterol represent two separate layers of a control mechanism in regulating reductase activity. The residual basal ACAT activity resistant to U18666A inhibition is analogous to cholesterol-resistant reductase activity. The partial inhibition by U18666A may have been due to the fact that more than one mechanism controls ACAT activity, and that only one of these is altered by U18666A. It may be noted that 150 ng/ml of U18666A was needed to achieve half of the maximal inhibition. This estimated value was calculated based on the fact that the maximal inhibition of U18666A was 80% (Fig. 1).

Cholesteryl ester apparently plays some role in the development of the brain as indicated by its transient accumulation in the immature brain. U18666A is effective in inducing epileptiform activity only in immature animals. This coincidence is consistent with the notion that the blockage of cholesteryl ester biosynthesis in the developing brain by U18666A may be the underlying defect in epileptic animals.

Drugs that inhibit the synthesis of cholesterol usually enhance cholesteryl ester formation [6, 7].

Table 2. Effect of U18666A on ACAT activity*

U18666A concn (μg/ml)	ACAT activity (cholesteryl ester formed)	
	pmoles·hr^{-1}·mg^{-1} (range)	%
0	1220 (254)	100
0.1 (in assay)	1530 (46)	125
1 (in assay)	1300 (32)	106
0.1 (in medium)	657 (178)	54
1 (in medium)	672 (101)	55

* The ACAT assay in cell-free homogenates was done according to the procedures described in Materials and Methods. U18666A was included directly in the assay mixture to determine its effect on ACAT activity in broken cells. For the experiments involving intact cells, the cells were incubated with U18666A for 5 hr and were washed with PBS prior to breaking up the cells for the determination of ACAT activity.

This is achieved by a coordinate regulation of 3-hydroxymethyl-3-glutaryl-CoA reductase and ACAT activities. U18666A represents one exception to this rule. This drug may be potentially useful for studying the biochemical consequences of an abnormal coordination between cholesterol formation and cholesteryl ester synthesis.

An *in vitro* study [11] indicates that there are two distinct steps in the metabolic pathway for cholesterol biosynthesis that are sensitive to U18666A. At low concentrations of the drug, desmosterol reductase is blocked [11, 18]. This leads to an abnormal accumulation of desmosterol [11]. As the concentrations of the inhibitor are increased, an earlier step in the cholesterol biosynthetic pathway is blocked. Although the enzyme affected has not been pinpointed, it was observed that U18666A blocks the incorporation of mevalonate into lanosterol and enhances the accumulation of squalene in this *in vitro* study. In addition, a recent report suggested that mevalonate is diverted to the biosynthesis of ubiquinone, another nonsterol isoprenoid, when U18666A interferes with cholesterol biosynthesis [13], although this finding appears to be controversial [19]. We now demonstrate an effect of U18666A on the formation of cholesteryl ester. Thus, the action of the drug is multi-faceted.

Cellular cholesterol biosynthesis supplies cholesterol for membrane needs. However, these needs can, alternatively, be met by exogenous lipids [20]. It may be argued that, if an adequate supply of cholesterol is available from extracellular sources, endogenous cholesterol biosynthesis is not essential. In fact, an elegant regulatory mechanism in mammalian cells [20] serves to inhibit cellular cholesterol biosynthesis by exogenous lipids contained in a lipoprotein complex. Since U18666A interferes with cholesterol esterification in cells grown in lipoprotein-containing medium, the lower rate of cholesterol esterification in U18666A-treated cells could not be due to cellular cholesterol deficiency.

In contrast to the multiplicity of cholesterol supply, there is only one route for the formation of cholesteryl ester. Cellular cholesteryl ester cannot be directly obtained from extracellular lipoproteins, because cholesteryl ester that is internalized in the form of lipoprotein complex is immediately hydrolyzed in the lysosomes. As a result, cholesteryl ester must be synthesized endogenously. Thus, a deficiency in cellular cholesteryl ester biosynthesis may be

detrimental to cells. The interference with cholesteryl ester formation by U18666A during a critical developmental period may be intimately associated with the adverse effects of U18666A. For example, cholesteryl ester was proposed to be involved in the storage of fatty acids [21]. The U18666A-induced deficiency in cholesteryl ester formation may result in a harmful accumulation of fatty acids. This represents one possible mechanism whereby U18666A interferes with myelinogenesis.

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