methods

Synthesis and biochemical properties of a new photoactivatable cholesterol analog 7,7-azocholestanol and its linoleate ester in Chinese hamster ovary cell lines

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Abstract **We report the chemical synthesis of a new photoactivatable cholesterol analog 7,7-azocholestanol (AC) and its linoleate ester (ACL). We also examined the biochemical properties of the sterol and its ester by employing several different mutant Chinese hamster ovary (CHO) cell lines with defined abnormalities in cholesterol metabolism as tools. AC mimics cholesterol in supporting the growth of a mutant cell line (M19) that requires cholesterol for growth. In normal cells, tritiated ACL present in low-density lipoprotein (LDL) was hydrolyzed and reesterified in a manner similar to tritiated cholesteryl linoleate (CL) in LDL. Also, in the mutant cell line (AC29) lacking the enzyme acyl-coenzyme A:cholesterol acyltransferase or in the mutant cell line (CT60) defective in the Niemann-Pick type C1 protein, the hydrolysis of ACL in LDL was normal, but the reesterification of the liberated AC was defective. Therefore, the metabolism of ACL in LDL is very similar to that of CL in LDL. Tritium-labeled AC delivered to intact CHO cells as a cyclodextrin complex was shown to photoaffinity label several discrete polypeptides, including caveolin-1. These results demonstrate AC as an effective reagent for studying cholesterol-protein interactions involved in intracellular cholesterol trafficking.**—Cruz, J. C., M. Thomas, E. Wong, N. Ohgami, S. Sugii, T. Curphey, C. C. Y. Chang, and T-Y. Chang. **Synthesis and biochemical properties of a new photoactivatable cholesterol analog 7,7-azocholestanol and its linoleate ester in Chinese hamster ovary cell lines.** ***J. Lipid Res.*** **2002.** 43: **1341–1347.**

Supplementary key words UV cross-linking • photoactivatable cholesterol analog • cholesterol-protein interaction • cholesterol mutants • Niemann-Pick type C disease • acyl-coenzyme A:cholesterol acyltransferase • caveolin-1

Biological membranes consist of lipids and proteins. To study lipid-protein interactions, an effective approach has been to affinity-label specific lipid binding proteins by employing UV cross-linkings using various radiolabeled, photoactivatable lipid molecules (1). For photolabile-steroid analogs, the preparation and properties of many steroid diaziridines and diazirines (which produce the carbene intermediates upon photolysis) were reported in the 1960s (2). Using the same synthetic procedures, Kramer and Kurz (3) reported the chemical synthesis of several photolabile analogs of bile salts suitable for photoaffinity labeling. Taylor et al. (4) reported the synthesis of labeled oxysterol 7,7-azo-cholestane-3β, 25-diol, and achieved successful labeling and identification of the oxysterol receptor in mouse L-cell extracts. Very recently, Thiele et al. (5) reported the chemical synthesis of a photolabile cholesterol analog 6,6-azocholestanol (also called photocholesterol), and its use in the identification of specific cholesterol binding proteins in neuronal cells (5). Photocholesterol has also been used to identify specific sterol binding proteins in *Caenorhabditis elegans* (6). The biophysical behavior of photocholesterol in model membranes has been shown to be very similar to that of cholesterol (7).

In mammalian cells, the major exogenous cholesterol source comes in the form of LDL. Normally, LDL contains cholesteryl linoleate (CL) as its major lipid cargo. LDL binds to the LDL receptor in the plasma membrane (PM); the complex then internalizes and enters the hydrolytic/lysosomal compartment where CL is hydrolyzed. The free cholesterol liberated from hydrolysis eventually enters an intracellular compartment that contains the Niemann-Pick type C1 protein (NPC1). From this compartment,

Abbreviations: AC, 7,7-azocholestanol; ACL, 7,7-azocholestanol linoleate; ACO, 7,7-azo-5α-cholestan-3β-ol oleate; BHT, 2,6-di-tert-butyl-*p*-cresol; CHO, Chinese hamster ovary; CL, cholesteryl linoleate; ER, endoplasmic reticulum; MβCD, methyl β-cyclodextrin; NPC1, Niemann-Pick Type C1; PCC, pyridinium chlorochromate.

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cholesterol recycles back to the PM, or moves to the endoplasmic reticulum (ER) for reesterification by the enzyme ACAT (8–12); (reviewed in 13). Whether photocholesterol could be incorporated into LDL, and if so, whether the fate of LDL-derived photocholesterol in mammalian cells is similar to that of LDL-derived cholesterol has not been examined. In this manuscript, we report the chemical synthesis of a new photoactivatable cholesterol analog 7,7-azocholestanol (AC) and its linoleate ester (ACL), as well as a method to label AC with ^{3}H at high-specific radioactivity. We also examined the biochemical properties of the sterol and its ester by employing several different mutant Chinese hamster ovary (CHO) cell lines with defined abnormalities in cholesterol metabolism as tools. The results show that the fate of LDL-bound ACL in CHO cells is very similar to that of LDL-bound CL. An additional result showed that labeled AC delivered to intact CHO cells as a cyclodextrin complex was able to cross-link several discrete polypeptides after UV-irradiation.

MATERIALS AND METHODS

General methods

Infrared Spectra were determined on a Perkin-Elmer spectrophotometer. ^{1}H-NMR spectra were determined on a 300 MHz Varian spectrometer, with deuteriochloroform as the solvent. UV spectroscopy was performed with a Gilford spectrometer with ethanol as the solvent. Composition analysis was performed at Atlantic Microlab, Norcross, GA. Organic solvents are from Aldrich, in HPLC grade whenever available. Unless stated otherwise, all chemical reagents used for synthesis were from Aldrich. The synthesis starting from 5-cholestan-3β-ol-7-one (I) to 7,7-azo-5α-cholestan-3α-[^{3}H]3β-ol (VI) ([^{3}H]AC) is outlined as follows. Unless stated otherwise, the purity of all the compounds synthesized was analyzed by TLC, and was found to be at least 90% pure.

5α-Cholestan-3β-ol-7-one (II)

5α-Cholestan-3β-ol-7-one (I) in the amount of 384.5 mg (0.96 mmol) was dissolved in 100 ml of anhydrous 2-propanol and slowly added to a vessel containing 86.5 mg of 10% palladium on carbon (from Eastman Kodak). The mixture was hydrogenated at room temperature for 110 min. The catalyst was removed by filtration through celite. Rotary evaporation removed the solvent, leaving flaky white crystals in nearly 100% yield.

5α-Cholestan-7,7-hydrazi-3β-ol (III) and 7,7-azo-5α-cholestan-3β-ol (IV)

Following the procedure of Church and Weiss (2), a solution of 266 mg (0.66 mol) ketone (II) was dissolved in 30 ml anhydrous methanol under nitrogen and cooled to 0°C. Dry ammonia was bubbled through for 2 h. From here on all the reactions were carried out in the dark. The mixture was stirred, and a solution of 350 mg (3.1 mol) hydroxylamine-*O*-sulfonic acid in 5 ml anhydrous methanol was slowly added over 20 min. The resulting cloudy mixture was stirred at 0°C for 1 h, then at room temperature for 5–12 h. The white precipitate formed was removed by filtration through a glass wool-plugged funnel and the filtrate was evaporated to give the diaziridine intermediate 5α-cholestan-7,7-hydrazi-3β-ol (III) as a white residue. The intermediate compound (III) was quickly dissolved in a mixture of 25 ml dry methanol and 1 ml triethylamine. While vigorously stirring, the mixture was treated with 400 mg iodine in 5 ml dry methanol so the slightly brown iodine color persisted. Sodium dithionite was slowly added to reduce the excess iodine. The reaction mixture was washed with saturated sodium chloride solution, then with water, dried over magnesium sulfate, then evaporated to dryness to yield a yellowish-white residue. TLC analysis indicated that the residue was a mixture of three or four organic components. Flash chromatography of the mixture in a 20 mm × 15 cm column of silica gel 60 using the solvent system hexane-ethyl acetate (1:2, v/v) yielded pure compound IV with approximately 60% yield.

7,7-Azo-5α-cholestan-3-one (V)

Following the procedure from Corey and Suggs (14), 90 mg (0.42 mmol) pyridinium chlorochromate (PCC) was suspended in 0.67 ml anhydrous dichloromethane. A solution of 115.2 mg 7,7-azo-5α-cholestan-3β-ol (IV) in 0.5 ml dichloromethane was added to the PCC mixture. The reaction mixture stood with stirring at room temperature for 1–2 h, then was diluted with 5 vol of anhydrous ethyl ether. The solvent was decanted and the residue washed twice with anhydrous ethyl ether. The organic phases were combined and filtered through Florisil. The filtrate was rotary evaporated and white crystals of compound (V) were obtained in nearly quantitative yield.

7,7-Azo-5α-cholestan-3α-[^{3}H]-3β-ol (VI)

Based on the procedure by Kramer and Kurz (3), a 1 M stock solution of 100 mCi NaB[^{3}H]$_4$ in H_2O (0.3783 mg, 10 μmol, specific activity: 5.2 Ci/mmol, from Amersham) was prepared; 10 μl was pipetted into the reaction vial and lyophilized. Next, 4.2 mg (10 μmol) of the 7,7-azo ketone (V) was dissolved in 400 μl ethanol and 30 μl H_2O. The ketone solution was added to the vial containing the solid NaB-[^{3}H]$_4$. The mixture was stirred for several minutes, then allowed to occur at room temperature for 2.5 h. The products consisted of the 3α-ol and the 3β-ol (VI) isomers. The 3α-ol isomer (as a byproduct of the reaction) migrated slightly faster than the 3β-ol isomer (VI) on TLC with hexane-ethyl acetate (1:2, v/v), with an R_f differential of approximately 0.1. The 3β-alcohol isomer (VI) was isolated in the pure form by extraction from the silica gel after TLC separation on a 20 × 20 cm preadsorbent 250 μm silica gel plate (from J. T. Baker). After extraction with 4 vol of methanol followed by 4 vol of dichloromethane, the solvent was rotary evaporated to yield [^{3}H]AC in ~60% yield. The purified compound was stored in methanol at −80°C. TLC analysis revealed that no detectable autoradiololysis of [^{3}H]AC (VI) occurred for at least 2 years. The non-radiochemical compound (VI) was synthesized in the same manner using nonradiochemical $NaBH_4$.

7,7-Azo-5α-cholestan-3α-[^{3}H]-3β-ol linoleate

Based on the procedure of Lentz et al. (15), the solvent (methanol) from an amber vial containing the purified [^{3}H]AC (1 mg, 2.4 μmol) was evaporated under nitrogen and resuspended in 88 μl benzene. An 80 μl of 100 mM linoleic anhydride in benzene (4.3 mg, 8.0 μmol) was added to the [^{3}H]AC solution. Two microliters of 0.1% 2,6-di-tert-butyl-*p*-cresol (BHT) in benzene (final concentration 0.001% BHT), and 30 μl of 1 mg/ml dimethyl amino pyridine in benzene (30 μg, 250 nmol) was added to the amber vial. The solution was briefly flushed with nitrogen, capped tight, and stirred overnight. The resultant crude product contained 7,7-azo-5α-cholestan-3β-[^{3}H]3α-ol linoleate ([^{3}H]ACL). The crude product was purified by preparative TLC using a preparative TLC Si1000 plate (J. T. Baker), developed in hexane-benzene (1:1; v/v). Non-radiochemical azoCL was synthesized by the same method using non-radiochemical AC and linoleic anhydride. To recover [^{3}H]ACL after TLC, non-radio-

chemical ACL was used as a standard in a parallel lane, and was visualized with 0.05% dichlorofluorescein (in ethanol). The appropriate band that contained [^{3}H]ACL was scraped off the plate and extracted with 150 ml 10% methanol in diethyl ether and filtered through a sintered glass funnel. The solvent was rotary evaporated to yield the purified azocholesteryl ester. The radiochemical purity determined by TLC analysis was consistently above 80% with a yield of approximately 60–70%. The slightly lower radiochemical purity of [^{3}H]ACL (less than 90%) is presumably due to the intrinsic instability of linoleate moiety present in ACL. For this reason, we routinely incorporate freshly synthesized [^{3}H]ACL into LDL (see below). The [^{3}H]ACL-LDL was used for various experiments within one week of its preparation.

7,7-Azo-5α-cholestan-3β-ol oleate

The same procedure for synthesizing ACL described above was used to synthesize 7,7-azo-5α-cholestan-3β-ol oleate (ACO; compound IX), using non-radiochemical AC and oleic anhydride. The yield was approximately 60–70%.

Cell lines

Wild-type (WT) CHO cells were from ATCC, and maintained in this laboratory for many years. The 25RA cells are CHO cells resistant to the cytotoxicity of 25-hydroxycholesterol (16) and contain a gain of function mutation in the SREBP cleavage activating protein SCAP (17). CT60 and CT43 cells are derived from 25RA cells (18), and lack the NPC1 protein (10). AC29 cells are derived from 25RA cells and lack acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1) (19, 20). M19 cells are derived from WT cells (21) and are defective in *S2P*, a gene encoding a specific protease required for intramembrane cleavages of SREBPs (22).

Other procedures

Cells were grown and cultured as described in the figure legends. LDL, [^{3}H]cholesteryl-linoleate labeled-LDL ([^{3}H]CL-LDL), [^{3}H]azocholesteryl-linoleate labeled-LDL ([^{3}H]ACL-LDL), delipidated serum, and methyl β-cyclodextrin (MβCD) complexes were prepared based on procedures previously described (10).

RESULTS

Chemical structure of AC

The chemical synthesis of AC and ACL were described in Materials and Methods. The characteristics of various intermediates/derivative-formed (compounds II, IV, V, and IX), including their R_f values in TLC analyses and their various spectrometric measures, are tabulated in **Table 1**.

The structures of AC (compound VI) and its predicted carbene intermediate (compound VII) upon UV irradiation are shown in **Fig. 1**. Two structural differences exist between cholesterol and AC:cholesterol and contain a Δ^5 double bond not present in AC; the hydrogen located at C-7 in cholesterol is replaced with a photoactivatable diazirine ring in AC. The diazirine ring in AC is located at C-7, instead of at C-6 as in photocholesterol (5).

AC can sustain the growth of a cholesterol auxotroph in cholesterol-free medium

We employed various assays to test if AC retains similar biological activities to cholesterol. WT CHO cells are able to grow indefinitely when grown in cholesterol-free medium. M19 cells are cholesterol auxotrophs that depend on the presence of exogenous cholesterol for growth (21). To determine if AC can serve as a sterol supplement for M19 cells, WT and M19 cells were incubated in cholesterol-free medium supplemented with cholesterol, or with AC, or with no sterol. The growth of the cells was monitored for 4 days. We found that WT cells grew in all medium conditions tested (**Fig. 2A**), whereas M19 cells grew in medium supplemented with cholesterol or with AC, but died in medium not supplemented with sterol (Fig. 2B). Only small differences in growth were observed when M19 cells were incubated with medium supplemented with either cholesterol or AC. These results indicate that AC can serve as a sterol source for growth in place of cholesterol in M19 cells.

TABLE 1. Characteristics of various compounds in synthetic pathway

Compound	TLC R_f	TLC Solvent	UV ε@ 350 nm	UV ε@ 367 nm	IR (cm^{-1}) Characteristic Absorption	^{1}H-NMR (ppm) Characteristic Maxima
II 5α-cholestan-3β-ol-7-one	0.285	Hexane/EtOAc (3:7)	n/a	n/a	1719 (C = O) (s[a]) 3370 (O – H) (s)	3.65 (m, 1, C-3); 0.65 (s, 3, C-18); 0.89/0.91 (d, 6, C-26/C-22); 0.93 (s, 3, C-21); 1.107 (s, 3, C-19); 2.33 (dt, 2, C-6); 2.19 (dq, 1, C-8)
IV 7,7-azo-5α-cholestan-3β-ol	0.491 0.303	Hexane/EtOAc (1:2) Hexane/EtOAc (2:1)	91.2	101.7	1572 (N = N) (m) 3462 (O – H) (s)	3.65 (m, 1, C-3); 0.025 (dd, 1, C-6); 0.3 (dq, 1, C-8); 0.93 (s, 3, C-19)
V 7,7-azo-5α-cholestan-3-one	0.740	Hexane/EtOAc (1:2)	n/a	n/a	1570 (N = N) (m) 1719 (C = O) (s)	0.025 (dd, 1, C-6); 0.125 (dq, 1, C-8); 1.18 (s, 3, C-19) [Note disappearance of alcohol at 3.65 ppm; also appearance of bands at 2.0–2.5 ppm which are α and β to carbonyl at C-3]
IX 7,7-azo-5α-cholestan-3β-ol oleate	0.790	Hexane/Benzene (2:3)	n/a	n/a	1574 (N = N) (m) 1652 (C = C) (w) 1733 (C = O) (s) 1000 (C – O) (m)	4.75 (m, 1, C-3); 5.38 (m, 2, C = C); 2.28 (t, 2, alpha to COO); 0.025 (dd, 1, C-6); 0.125 (dq, 1, C-8); 1.30 (d, 3, C-19 and CH2 in alkane of oleate)

[a] s, strong; m, medium; w, weak.

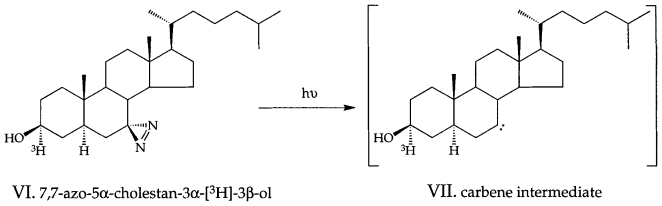

Fig. 1. Chemical structures of 7,7-azocholestanol (AC) (compound VI) and its carbene intermediate (compound VII) upon photolysis.

LDL-bound ACL can be hydrolyzed and reesterified in intact CHO cells

In order to determine if AC can be metabolized in intact cells, we utilized [^{3}H]ACL-LDL to examine the fate of the cholesterol analog in the LDL receptor pathway, taking advantage of additional CHO cell mutants. CT60 and CT43 cells, derived from 25RA cells (16), are NPC1 mutants defective in trafficking LDL-derived cholesterol to the ER for reesterification (10) by a resident ER protein, ACAT1 (23). AC29 cells, also isolated from 25RA cells, are mutants lacking ACAT1 (24). When we labeled 25RA, CT60, and AC29 cells with [^{3}H]ACL-LDL, we found that the [^{3}H]ACL-LDL was hydrolyzed to [^{3}H]AC in all cells (**Fig. 3A**). The hydrolyzed [^{3}H]AC was reesterified into [^{3}H]ACO in 25RA cells but no significant radiolabeled ester product was formed in CT60 or in AC29 cells (Fig. 3B). When directly comparing the metabolism of [^{3}H]ACL-LDL to [^{3}H]CL-LDL in 25RA cells, we found that [^{3}H]ACL-LDL is hydrolyzed similarly to [^{3}H]CL-LDL (Fig. 3C). Although [^{3}H]ACL-LDL can be reesterified in 25RA cells (Fig. 3B), the amount of [^{3}H]ACO formed is

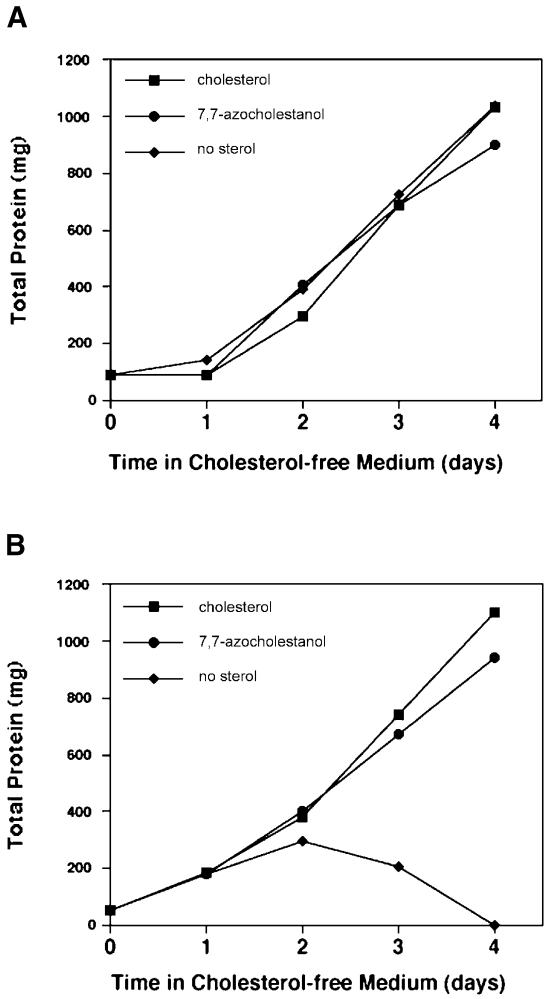

Fig. 2. AC can serve as a sterol source for cholesterol auxotroph M19 cells. A: WT and M19 (B) cells were plated in 25 cm^2 flasks in medium A (Ham's F-12, 10% FBS) as monolayers at 37°C with 5% CO_2. After 2 days, cells were washed several times with PBS and incubated with medium D (Ham's F-12 with 10% delipidated FBS, 35 μM oleic acid) in the presence or absence of 5 mg/ml exogenous sterol for the indicated times at 37°C before determining the total cellular protein content of each flask.

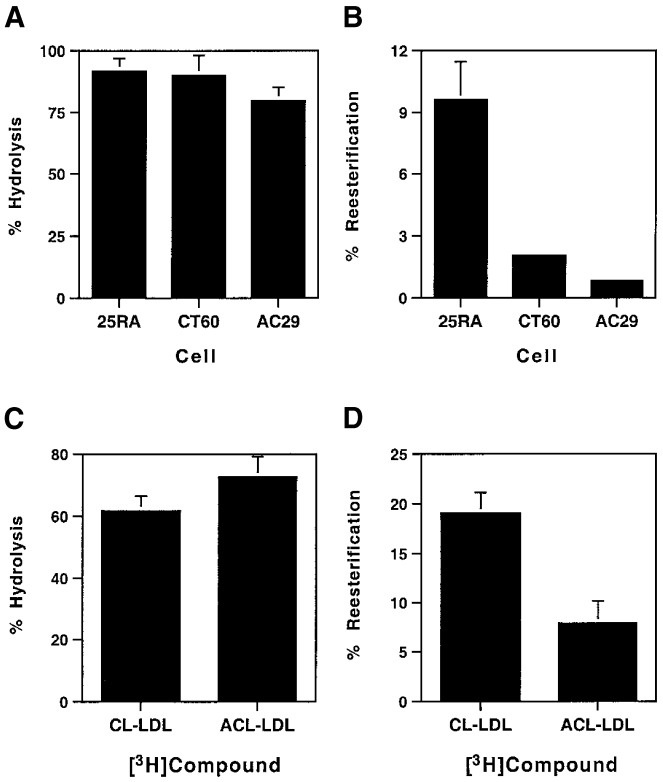

Fig. 3. [^{3}H]7,7-Azocholestanol linoleate (ACL)-LDL can be metabolized in intact Chinese hamster ovary (CHO) cells. A, B: 25RA, CT60, and AC29 cells were plated at 2 × 10^5 cells/well in 6-well dishes in medium A for 24 h at 37°C, washed several times with PBS, and incubated with medium D for 48 h at 37°C. Cells were pulse-labeled with 100 μg/ml [^{3}H]ACL-LDL in medium D for 6 h at 37°C. The cell extract was harvested, the cellular lipids were extracted and analyzed by TLC, and the percentage hydrolysis (A) and percentage reesterification (B) were determined as previously described (19), except that the internal TLC standards were AC, ACL, and AC oleate (ACO). C, D: 25RA cells were grown and cultured as described above but were pulse-labeled with either 100 μg/ml [^{3}H]CL-LDL or with 100 μg/ml [^{3}H]ACL-LDL in medium D for 6 h at 37°C before measuring the percentage hydrolysis (C) and percentage reesterification (D). The results shown are the averages of triplicate dishes and are representative of either three (A, B) or two (C, D) independent experiments. Error bars indicate the sizes of 1 SD.

less than the [^{3}H]cholesteryl oleate formed (Fig. 3D). We believe the reason for this lies in the substrate specificity of ACAT1. This is supported by the finding that although AC can serve as a sterol substrate for ACAT as determined by a reconstituted in vitro ACAT assay, cholesterol is still a better substrate than AC for ACAT1 (results not shown).

Cyclodextrin/AC complex can be employed for photolabeling proteins in intact cells

One approach to deliver AC to intact cells is via cyclodextrins, which are water soluble, cyclic oligomers of glucose that have the capacity to bind cholesterol within its hydrophobic core (25). We prepared MβCD complexes composed of either [^{3}H]cholesterol or [^{3}H]AC. We then incubated the WT CHO cells with [^{3}H]sterol alone, or with [^{3}H]sterol as MβCD complex at 37°C, then determined the total amount of incorporation of [^{3}H]sterol into the cells. We found that the cellular incorporations of [^{3}H]cholesterol or [^{3}H]AC were similar; the incorporations were much greater when delivered as a complex with MβCD than without MβCD (**Fig. 4**).

To test if [^{3}H]AC can cross-link proteins after UV photoactivation, we treated intact 25RA and CT43 cells with [^{3}H]AC/cyclodextrin complex for a brief period (1 h at room temperature) followed by UV exposure, and found that several discrete protein bands could be identified by SDS-PAGE-radioluminography (**Fig. 5A**). A total of eight discrete bands (indicated by arrows a–h, Fig. 5A), could be identified by this method. The estimated molecular weights (in kDa) of these bands are: a, 88; b, 68; c, 51; d, 38; e, 33; f, 21; g, 18; h, 11.7. The labeling patterns in the 25RA cells and the CT43 cells were very similar (comparing lanes 1 and 3 of Fig. 5). The limitation in sensitivity and resolution of the current method did not allow us to conclude whether or not the NPC1 protein, a membrane glycoprotein with apparent molecular weight of 150–180 kDa (13), or the ACAT1 protein, a membrane protein with apparent molecular weight of 45 kDa (20), was photolabeled. When an excess amount of unlabeled cholesterol was included during the labeling period, the labeling intensity for each of these bands significantly decreased (i.e., comparing results in lanes 1 and 3 vs. those in lanes 2 and 4 of Fig. 5). These results suggest that most or all of the labeled proteins recognize cholesterol in a specific manner. Based on Western blotting results (Fig. 5B), one of the labeled bands, the 20–21 kDa band, is probably caveolin-1. To positively reveal the identity of this band, after photolabeling the 25RA cell lysates were solubilized in detergent, and were immunoprecipitated either with mouse monoclonal antibody against caveolin-1 or with control mouse IgG; the immunoprecipates and the immunodepleted supernatants were analyzed by SDS-PAGE-radioluminography. The result (**Fig. 6**) showed that the major labeled band (band f) is caveolin-1, a known cholesterol binding protein. Each of these bands shown

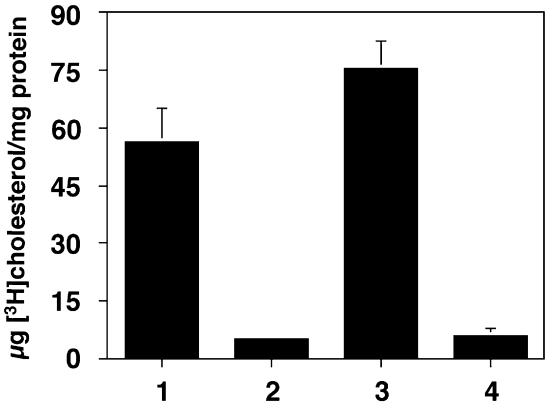

Fig. 4. Cyclodextrin can be used as an effective delivery vehicle to deliver [^{3}H]AC to intact CHO cells. WT cells were plated in 6-well dishes and grown in medium A at 37°C until cells were approximately 80–90% confluent. Cells were washed several times with PBS and incubated with 5 mM methyl β-cyclodextrin (MβCD):[^{3}H]cholesterol (lane 1), [^{3}H]cholesterol (lane 2), 5 mM MβCD:[^{3}H]AC (lane 3), or [^{3}H]AC (lane 4) in medium D for 7 h at 37°C. The same amount of total radioactivity was added to each dish. The cell extract was harvested, and the cellular lipids were extracted and analyzed on TLC as previously described (10). Results shown are the averages of triplicate dishes with error bars indicating the sizes of 1 SD.

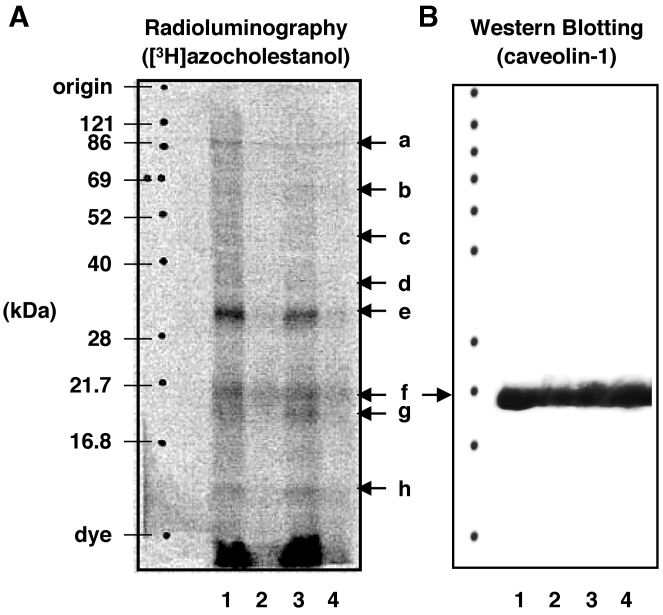

Fig. 5. Intact cell photoaffinity labeling of 25RA and CT43 cells using [3H]AC/cyclodextrin complex. A: 25RA (lanes 1, 2) and CT43 cells (lanes 3, 4) (1×10^6 cells, suspended in 0.1 ml of PBS) grown in medium A were photolabeled using [^{3}H]AC/cyclodextrin complex (0.32 μg sterol, 0.5 μCi) in absence (lanes 1, 3) or presence (lanes 2, 4) of 300-fold excess of unlabeled cholesterol. After incubation for 1 h at room temperature, the photolysis was conducted for 15 min at 4°C, using the complete photochemical reaction assembly (from Ace Glass, Inc) with a 450 watt UV lamp. After photolysis, the [^{3}H]AC labeled cells were washed with PBS, centrifuged at 4°C to remove unbound [^{3}H]AC. The cell-pellets were lysed using 10% SDS, and were subjected to 10% SDS-PAGE, followed by radioluminography (27) (exposure time: 5 days). B: In parallel with radioluminography, after SDS-PAGE, the parallel set of samples was immunoblotted with mouse monoclonal anti-caveolin-1 antibody (Transduction Laboratories, clone No. 2234). The estimated molecular weights (in kDa) of the labeled bands are: a, 88; b, 68; c, 51; d, 38; e, 33; f, 21; g, 18; h, 11.7.

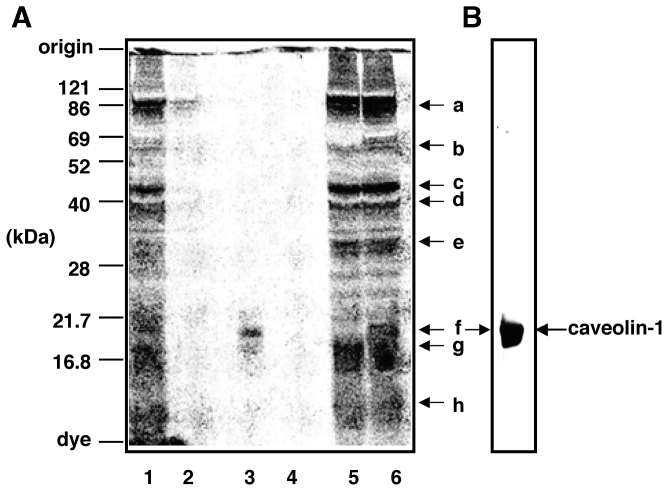

Fig. 6. Immunoprecipitation of photolabeled caveolin-1 in 25RA cells. A: 25RA cells grown in medium D for 48 h [2×10^6 cells, suspended in 0.2 ml of Hanks' buffer (pH 7.4)] were photolabeled by incubating with [^{3}H]AC/cyclodextrin complex (0.32 μg, 8.8 μCi) for 1 h at 37°C, followed by photolysis for 15 min on ice. Unless stated otherwise, the photolabelings were carried out in the absence of excess unlabeled cholesterol. The [^{3}H]AC labeled cells were centrifuged to remove unbound [^{3}H]AC. The cell-pellets were lysed using lysis buffer (50 mM Tris (pH 7.4), 100 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and 500-fold diluted protease inhibitors cocktail (Sigma). The supernatant was collected by centrifugation at 10,000 *g* for 15 min at 4°C. The lysates were subjected to immunoprecipitations using mouse monoclonal anti-caveolin-1 or control mouse IgG according to procedure described (28, 29). The immunoprecipitates and the immunodepleted supernatants were subjected to 10% SDS-PAGE, followed by radioluminography (exposure time: 5 days). Lane 1: [^{3}H]AC-labeled lysate before immunoprecipitation; lane 2: same as lane 1 except photolabeling was carried out in the presence of 100-fold excess of unlabeled cholesterol; lane 3: immunoprecipitate of [^{3}H]AC-labeled lysate, using mouse monoclonal anti-caveolin-1 (Transduction Laboratories, clone No. 2234); lane 4: immunoprecipitate of [^{3}H]AC-labeled lysate, using control mouse IgG; lane 5: immunodepleted supernatant of [^{3}H]AC-labeled lysate, after immunoprecipitation using anti-caveolin-1; lane 6: immunodepleted supernatant of [^{3}H]AC-labeled lysate, after immunoprecipitation using control mouse IgG. B: In parallel with radioluminography, after SDS-PAGE, the parallel set of samples was immunoblotted with rabbit polyclonal anti-caveolin-1 antibodies (Santa Cruz: N-20).

in Figs. 5 and 6 could be composed of a single polypeptide, or multiple polypeptides with overlapping molecular weights. In the future, the immunoprecipitation strategy described here coupled with extensive purification of individually labeled bands may help to reveal the identity of these labeled proteins other than caveolin-1.

DISCUSSION

In this manuscript, we reported the chemical synthesis of a new photoactivatable cholesterol analog AC labeled with ^{3}H at high-specific radioactivity, and described methods to synthesize its oleate or linoleate ester. The procedures described can be carried out in standard biochemical laboratories. We then took a cell biological approach to examine the biochemical properties of AC and its ester by employing several different mutant CHO cell lines as tools. The results showed that AC can substitute for cholesterol as a sterol source in a cholesterol auxotroph. When delivered to intact cells as [^{3}H]ACL-LDL, [^{3}H]ACL is hydrolyzed into free [^{3}H]AC in the hydrolytic/lysosomal compartment, transported to the ER via an NPC1-mediated pathway, and reesterified into [^{3}H]azocholesteryl oleate by ACAT1. Thus, AC faithfully mimics cholesterol in terms of the LDL receptor mediated metabolic pathway in intact cells. As described in Fig. 5, labeling intact 25RA cells with [^{3}H]AC/cyclodextrin complex followed by UV irradiation enabled us to identify several candidate cholesterol-binding proteins. Based on Western analysis and immunoprecipitation analyses, one of these proteins is caveolin-1. We are currently pursuing the identification of these other candidate cholesterol-binding proteins. We also showed that LDL-bound [^{3}H]ACL is metabolized in a similar manner to LDL-bound [^{3}H]CL. Thus, in the future, AC and its ester may also serve to identify novel cholesterol binding proteins involved in the LDL receptor mediated pathway in mammalian cells. Regarding the relative reactivity between AC and the 6,6-azocholestanol (photocholesterol) (5): based on the well-known conformation of cholestanol (26), the insertion of the carbene at C-6 of steroid into any given protein may create axial-axial interaction between the angular methyl group at C-8 of the steroid, and the axial carbon-carbon bond formed between the steroid and the protein. Such an interaction does not occur if the carbene is located at C-7. Therefore, AC may be a more efficient photoaffinity-labeling reagent than 6,6-azocholestanol. This possibility can be tested by future experimentation.

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