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Interactions of a photosensitive analog of cholesterol with hydroxymethylglutaryl-CoA reductase (NADPH) and acyl-CoA:cholesterol acyltransferase

(lipid–protein interaction/membrane-bound enzymes/photosensitive probes/25-azidonorcholesterol)

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ABSTRACT **25-Azido[25-^{3}H]norcholesten-3β-ol, a radioactive photosensitive analog of cholesterol, was synthesized as a probe to study lipid–protein interactions [Stoffel, W. & Klotzbücher, R. (1978) *Hoppe-Seyler's Z. Physiol Chem.* 359, 199–209]. Upon UV irradiation a reactive nitrene is generated which can insert into carbon–hydrogen bonds of proteins to form covalent linkages. The compound has properties similar to those of cholesterol. When administered intravenously to rats it was transported to the liver, and within 4 hr it was found in all of the subcellular fractions, accounting for 8% of the microsomal sterol content. Concomitantly, it altered the activities of two membrane-bound enzymes. 3-Hydroxy-3-methylglutaryl (HMG)-CoA (NADPH) reductase [mevalonate:NADP$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34] activity was reduced to 20% of control values, and acyl-CoA:cholesterol acyltransferase (ACAT; acyl-CoA:cholesterol *O*-acyltransferase, EC 2.3.1.26) was stimulated at least 2-fold. Esterified products of the analog were found in both blood and liver, and no evidence was obtained for production of any hydroxylated or polar derivatives. UV irradiation of microsomes increased the radioactivity bound to the protein fraction and decreased it in the total lipid extract; in parallel a sharp decline in ACAT activity but unaltered HMG-CoA reductase activity was observed. These results are interpreted as a possible consequence of crosslinking of the sterol to the enzyme proteins and discussed in view of the evidence that HMG-CoA reductase is an extrinsic and ACAT an intrinsic membrane protein.**

Radiolabeled lipids containing azido groups have recently been introduced as probes to study lipid–protein interactions (1–4). Azido-labeled lipids can be easily incorporated into biological membranes and then photoactivated to generate reactive nitrenes, which during their short lifetime (10^{-11} sec) can react by addition to double bonds or by insertion into C—H bonds of neighboring lipids and proteins to form covalent crosslinks. The incorporation into membranes of azido phospholipids or azidocholesterol followed by their photoactivation and analyses for lipid covalently linked to membrane proteins provides a means of determining the topography of lipids neighboring a particular membrane protein. In the case of membrane-bound enzymes the influence of crosslinks on enzyme function can also be measured. Fatty acids containing azido groups at different positions in the acyl chain have been synthesized and incorporated into lysolecithin, phospholipids, sphingomyelin, and cholesterol esters. These lipids have been used in the photoactivated crosslinking reactions to provide insight into the spatial arrangement of the lipid and proteins of high density lipoprotein (2). Recently similar techniques have been applied to study the localization of the G and M proteins of vesicular stomatitis virus (4).

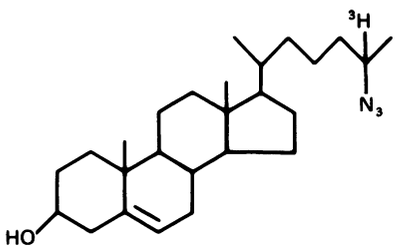

FIG. 1. 25-Azido[25-^{3}H]norcholesten-3β-ol.

The azido group has also been introduced into the C-25 side chain position of cholesterol. 25-Azido[25-^{3}H]norcholesten-3β-ol (azido[25-^{3}H]norcholesterol) was synthesized and shown to be incorporated into BHK-21 (baby hamster kidney 21) cells as a cholesterol substitute without any growth impairments. Similarly to cholesterol, azidonorcholesterol inhibited de novo sterol synthesis as well as 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (NADPH) [mevalonate:NADP$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34] activity, while it did not affect the incorporation of mevalonate into sterols (3). Consequently, azidonorcholesterol is ideally suited to probe the interaction of sterols with membrane proteins, particularly HMG-CoA reductase. Another well-suited enzyme for such a study is acyl-CoA:cholesterol acyltransferase (ACAT; acyl-CoA:cholesterol *O*-acyltransferase, EC 2.3.1.26), because whereas HMG-CoA reductase is an extrinsic membrane enzyme (5), ACAT is most likely intrinsic (6) and catalyzes the esterification of cellular cholesterol. Both enzymes are involved in sterol metabolism and their enzymatic activities are reciprocally coordinated (7): increase in cellular cholesterol causes a dramatic decrease in HMG-CoA reductase activity, whereas it increases ACAT activity. In this report we describe the effects of administration of 25-azido[25-^{3}H]norcholesterol to rats and the effects of its photoactivation on the activities of HMG-CoA reductase and ACAT.

MATERIALS AND METHODS

The synthesis of 25-azido[25-^{3}H]norcholesten-3β-ol from 26-norcholesten-3β-ol-25-one and criteria establishing its purity have been described in detail by Stoffel and Klotzbücher (3). The chemical structure of the compound is shown in Fig. 1. The

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Abbreviations: azidonorcholesterol, 25-azidonorcholesten-3β-ol; HMG, 3-hydroxy-3-methylglutaryl; ACAT, acyl-CoA:cholesterol acyltransferase.

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compound was administered intravenously to animals in a phospholipid dispersion made by sonicating 5 mg of azidonorcholesterol with 45 mg of soya lecithin in 1 ml of phosphate-buffered saline. During sonication the sample was kept on ice and under a stream of argon. It was filtered through a 45-μm Millipore filter before injection into the tail vein of rats. The animals had been maintained on a light–dark cycle of lights on from 8 p.m. to 8 a.m. and were injected 4 hr before the induction of maximum reductase activity—i.e., 10 a.m. They were sacrificed 4 hr later, their livers were excised, and the microsomal fraction was prepared according to previously published procedures (5).

Radiolabeled compounds were purchased from New England Nuclear. Sources for other supplies were the same as published previously (5). HMG-CoA reductase activities were determined by the microassay described by Shapiro *et al.* (8). Each assay contained 25 nmol of [3-^{14}C]HMG-CoA [0.9 μCi/μmol (1 Ci = 3.7 × 10^{10} becquerels)]. To calculate extraction efficiencies [5-^{3}H]mevalonate (0.08 μCi per assay) was used as the internal standard. ACAT was assayed by modifications of methods described by Brown *et al.* (9) and Brenneman *et al.* (10). A total assay volume of 0.5 ml contained 1.9 mg of bovine serum albumin; 10 mM dithiothreitol; 0.5–1.0 mg of microsomal protein; 50 mM KH_2PO_4 buffer, pH 7.4; and 30 nmol of [1-^{14}C]palmitoyl-CoA (2.2 μCi/μmol).

Endogenous microsomal cholesterol was used as the other substrate. The mixture was preincubated at 37°C for 5 min before the addition of [^{14}C]palmitoyl-CoA and the enzyme was assayed for 10 min. The reaction was stopped with 2 ml of $CHCl_3/CH_3OH$ (2:1, vol/vol) mixture and cholesterol and cholesterol esters were isolated from the total lipid extract by thin-layer chromatography on silica gel, using hexane/diethyl ether/methanol/acetic acid (180:40:4:6, vol/vol) as the solvent. Recoveries were quantitated with [^{3}H]cholesterol palmitate (0.03 μCi per assay) added as the internal standard. Under these reaction conditions ACAT showed linear kinetics (R. A. Heller and R. D. Simoni, unpublished data).

Sterols were isolated as total lipid extracts with $CHCl_3/CH_3OH$ (2:1) and then quantitated by gas/liquid chromatography on 1% OV 17 (Supelco, Bellefonte, PA) columns at 250°C. Azidonorcholesterol decomposes under these conditions and therefore did not contribute to the cholesterol content. It was separated by thin-layer chromatography on silica gel in petroleum ether/diethyl ether/methanol (70:30:7, vol/vol), in which it migrated with an R_F of 0.61. Azidonorcholesterol esters migrated with the same R_F as cholesterol esters. They were eluted with $CHCl_3$ and hydrolyzed by mild alkaline hydrolysis in methanolic KOH and rechromatographed to estimate the amount of [^{3}H]azidonorcholesterol. In this solvent system polar compounds such as hydroxylated derivatives of cholesterol remained at the origin.

Irradiation conditions to photoactivate azidonorcholesterol were as follows: Microsomal suspensions (1.5–2.0 mg of protein per ml) were deoxygenated by short periods of evacuation followed by purges with argon, four to six times per hr for 2 hr. The sample was then placed in a water bath at 4°C and exposed for different periods of time to UV irradiation at wavelengths above 300 nm from a Philips Mittelhochdruck mercury lamp kept in a Duran glass vessel that served as a cutoff filter for wavelengths below 305 nm. Aliquots of microsomal suspension were removed at intervals and assayed for enzyme activities.

The distribution of azidonorcholesterol in the lipid and protein fractions of microsomes was determined in 100-μl or 400-μl aliquots that had been lyophilized and then extracted twice with 3 ml of $CHCl_3/CH_3OH$ (1:1). After centrifugation the extracted lipids in the supernatant were evaporated to dryness and redissolved in 100 μl of $CHCl_3$, and the radioactivity was determined in Instagel scintillation fluid. The protein pellet was first solubilized in 500 μl of Soluene-350 (Packard) and then used for the determination of its radioactivity content.

Table 1. Distribution and content of azidonorcholesterol in liver subcellular fractions

Fraction	Content, μg/mg protein: Azidonorcholesterol	Content, μg/mg protein: Cholesterol	Azidonorcholesterol, % of total
Liver homogenate	1.93	12.2	16
10,000 × *g* pellet	1.99	21.8	8
100,000 × *g* pellet	1.87	21.6	8
100,000 × *g* supernatant	1.12	1.1	50

RESULTS

Because 25-azido[25-^{3}H]norcholesterol substituted for the cholesterol requirement of BHK-21 cells and, like cholesterol, it inhibited cellular sterol synthesis by inhibition of HMG-CoA reductase activity (3), further investigations were conducted to see whether intravenous administration of azidonorcholesterol to rats had similar effects. HMG-CoA reductase activity of rat liver microsomes assayed 4 hr after a single dose of 5 mg of azidonorcholesterol per animal showed a dramatic decline: in four different animals, down to 18% ± SD 4.5% of noninjected controls. Analyses for azidonorcholesterol proved its presence in the different subcellular fractions of the liver. In the microsomal fraction it amounted to 8% of the sterol content (Table 1). Resolution into free and esterified fractions revealed less than 3% of the microsomal content to be esterified (Table 2).

In order to detect formation of any polar derivatives of azidonorcholesterol, thin-layer chromatographic analyses using acetic acid/carbon tetrachloride/diisopropyl ether/isoamyl acetate/1-propanol/benzene (5:20:30:40:10:10, vol/vol) and petroleum ether/diethyl ether/acetic acid (70:30:1, vol/vol) solvent systems were employed. Total lipid extracts of liver tissue or microsomes 4 and 7 hr after intravenous administration of azidonorcholesterol showed no metabolic radioactive products that corresponded to hydroxylated derivatives and no radioactivity coincident with the location of standard polar compounds such as cholic acid, lithocholic acid, and taurocholic acid. The radioactivity was limited to the original compound or its esters.

The blood sterol fraction also contained azidonorcholesterol. Assuming a blood volume of 18 ml per animal, the azidonorcholesterol content was estimated to be 1.6 mg; of this more than 50% was esterified.

Table 2. Distribution of azidonorcholesterol and azidonorcholesterol esters in subcellular fractions of rat liver

Fraction	Azidonorcholesterol, %	Azidonorcholesterol esters, %
Liver homogenate	89.3	10.7
10,000 × *g* pellet	93.0	7.0
100,000 × *g* pellet	97.7	2.3
100,000 × *g* supernatant	79.5	20.5

The conversion of azidonorcholesterol to azidonorcholesterol esters in the blood and liver implied the action of lecithin cholesterol acyltransferase and ACAT in blood and liver, respectively. Examination of the microsomal ACAT activity showed that it was stimulated at least 2-fold in azidonorcholesterol-injected animals compared to noninjected controls.

The above experiments define a system ideal for studying the interaction of sterols with membrane-bound enzymes; i.e., it uses a photoreactive sterol that is readily incorporated into membranes as a cholesterol substitute and has similar influences as cholesterol on two membrane-bound enzymes of sterol metabolism.

The next set of experiments describes the effects of photoactivation of azidonorcholesterol in the microsomal membranes. Optimal conditions for the UV irradiation were developed to prevent losses of enzymatic activities. Oxygen contained in the microsomal suspension was removed (2, 11), and irradiation at wavelengths above 300 nm was carried out as described in *Materials and Methods*. Under these conditions the ACAT and HMG-CoA reductase activities of microsomes not containing the azido compound remained unaltered.

In order to obtain evidence for crosslinking of photoactivated azidonorcholesterol to membrane proteins, analyses of its distribution between the lipid and protein fractions were conducted after 30, 60, 90, and 120 min of irradiation. Simultaneously, HMG-CoA reductase and ACAT were also assayed.

The distribution of azidonorcholesterol in the lipid and protein fractions of microsomal membranes as the photoactivation time was increased is presented in Fig. 2. The amount of probe in the lipid fraction declined with increased irradiation time, whereas the probe's binding to the protein fraction increased. The initial 30–60 min were most effective. It is apparent from these results that the sum of radioactivity in the two fractions even after 30 min of irradiation is less than that present at the start of the experiment. The loss of ^3H from the α-carbon (C-25) of the sterol side chain has been observed in irradiated samples of azidonorcholesterol phospholipid vesicles and is believed to be a result of the production of derivatives such as 25-keto or 25-imino compounds. The loss of ^3H increased with irradiation time, suggesting an increase in the production of these derivatives.

The ACAT activity in sterol-injected animals was at least 2-fold higher than in control animals. Photoactivation of the microsomes containing azidonorcholesterol for a 30- to 60-min period resulted in rapid loss of activity, which was unaffected by a longer time of irradiation (Fig. 3). It is possible that the drop in ACAT activity was a result of the formation of crosslinks between azidonorcholesterol and membrane cholesterol, which would diminish the amount of available substrate for the enzyme. Additional assays were therefore conducted in which exogenous cholesterol (50 μg in 5 μl of acetone) was added to each ACAT assay. It had been reported earlier (17) that sterol added in this form was utilizable by ACAT. However, supplementation with cholesterol did not alter the activity profile.

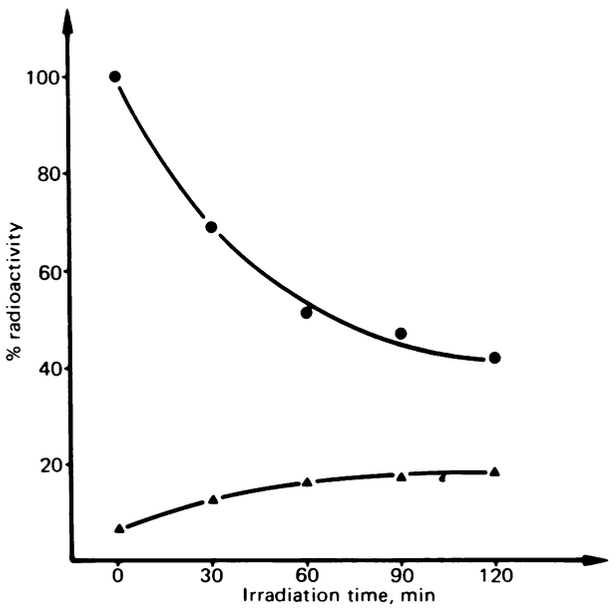

FIG. 2. Distribution of 25-azido[25-^3H]norcholesten-3β-ol in the lipid (●) and protein (▲) fractions of microsomal membranes as a function of photoactivation time. Deoxygenated microsomal suspensions (1.5–2.0 mg of protein per ml) were exposed to UV irradiation above 300 nm for increasing lengths of time and 400-μl aliquots were removed at regular intervals. These were twice extracted with 3 ml of $CHCl_3/CH_3OH$ (1:1) mixture, and the protein was pelleted by centrifugation. The pellet was digested in 500 μl of Soluene-350. The $CHCl_3/CH_3OH$ extracts were pooled, evaporated to dryness, and redissolved in $CHCl_3$. The ^3H contents of both lipid and protein fractions were measured in Instagel.

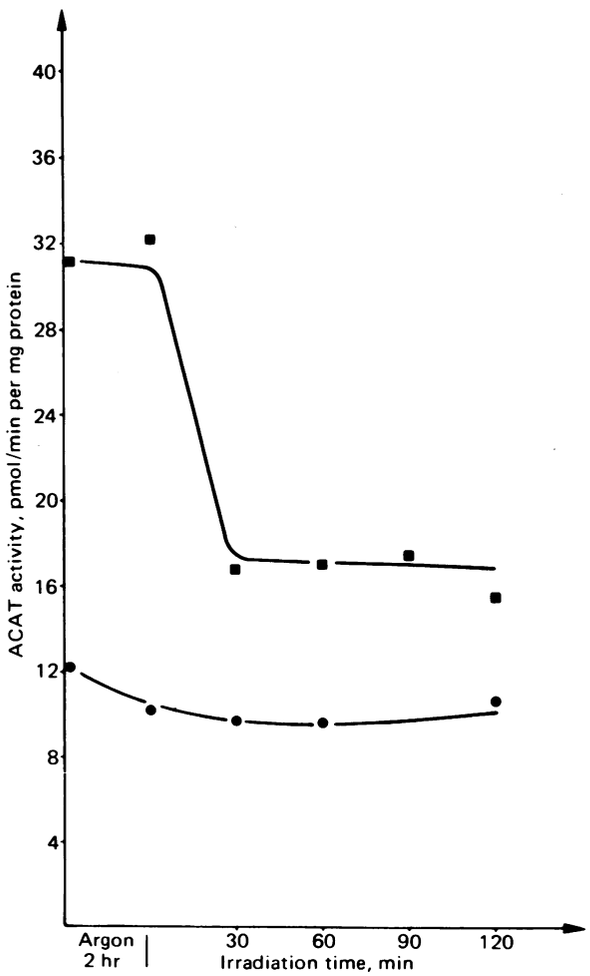

FIG. 3. ACAT activity in UV-irradiated microsomes obtained from uninjected control (●) and 25-azido[25-^3H]norcholesten-3β-ol-injected (■) animals. Microsomal suspensions (1.5–2.0 mg of protein per ml) were deoxygenated with argon for 2 hr and then UV irradiated for increasing periods of time. Aliquots (200 μl) were removed at regular intervals and assayed for ACAT. Duplicate samples were assayed and the mean activity is plotted.

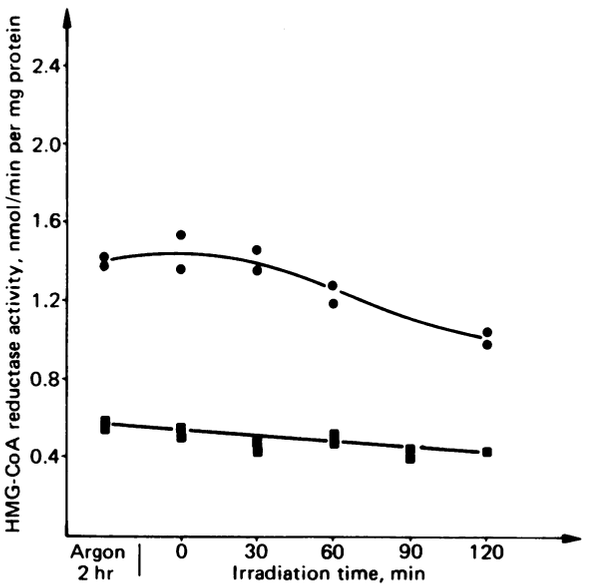

FIG. 4. HMG-CoA reductase activity in microsomes of uninjected control (●) and 25-azido[25-^{3}H]norcholesten-3β-ol-injected (■) animals as a function of irradiation time. Microsomal suspensions (1.5–2.0 mg of protein per ml) were deoxygenated and purged with argon for 2 hr, and the samples were UV irradiated for increasing periods of time. Aliquots (50 μl) were removed at regular intervals and assayed for HMG-CoA reductase. Duplicate samples were assayed and all the data points are plotted.

The HMG-CoA reductase activity of azidonorcholesterol-containing microsomes was reduced to 20% to start with. Photoactivation of these microsomes caused no further decline in activity, so that HMG-CoA reductase was unaffected under these conditions (Fig. 4).

DISCUSSION

This communication describes the use of 25-azidonorcholesterol, a photosensitive probe that effectively inhibits HMG-CoA reductase activity and consequently sterol synthesis, in a manner similar to that of cholesterol, and appears to be nontoxic. The compound is not converted to polar derivatives in rat liver, a reaction that has been reported to occur with oxygenated derivatives of cholesterol (12). 25-Azidonorcholesterol is incorporated into cellular membranes and also esterified intracellularly. Furthermore, microsomal ACAT is stimulated so that the reciprocal relationship between HMG-CoA reductase and ACAT is observed again (7). Azidonorcholesterol ester concentration is, however, highest in the cell cytosol and almost negligible in the microsomes at a time when the HMG-CoA reductase activity is reduced to 20%, so that under these conditions it appears unlikely that the cholesterol ester content of the microsomes is the effector that regulates HMG-CoA reductase activity, in contrast to some recent proposals (13, 14).

Interactions of membrane sterol with membrane protein have been reported, but examples were limited in number (15). To further investigate this association, especially that between cholesterol and HMG-CoA reductase or ACAT, 25-azidonorcholesterol appeared to be a suitable probe. In the liver, after the compound's intravenous administration, it was found to be distributed into all subcellular fractions. Its incorporation into microsomal membranes was also accompanied by changes in the catalytic function of two microsomal enzymes, HMG-CoA reductase and ACAT. The results of its photoactivation upon ACAT function suggest that it is present in close vicinity to the enzyme and that the decrease in ACAT activity may be due to crosslinking of the sterol to it. Because ACAT appears to be an integral membrane protein, it must interact with the lipid bilayer over much of its surface (6), and the effect of crosslinking can be seen as an immobilization or anchoring of the enzyme, thereby incapacitating it due to restrictions imposed upon it. The inhibition of ACAT by photoactivation could alternatively be interpreted by the photocrosslinking of the photosensitive substrate, the product, or both, to the active center of the enzyme. It is unlikely that the reduction in activity could be due to nonavailability of substrate because microsomal cholesterol should still be available even though some cholesterol–azidonorcholesterol crosslinks can be expected. Furthermore, exogenous cholesterol does not restore ACAT activity.

In azidonorcholesterol-injected animals HMG-CoA reductase was reduced to 20%, and photoactivation of the probe in the microsomes had no further effect on the activity. It should be emphasized that at this stage no experimental evidence is yet available for the direct interaction of 25-azidonorcholesterol with HMG-CoA reductase. It is a peripheral protein and its active side is exposed to the cell cytosol (5). In a reconstituted system the isolated enzyme showed interaction with phospholipid head groups and was insensitive to changes in the hydrocarbon chain, indicating interaction only at the bilayer surface (16). The localization of the rigid sterol nucleus of cholesterol is believed to be parallel to the phospholipid molecules, with the 3-OH group located close to the surface of the membrane and the sterol side chain extending into the membrane.

Applied to 25-azidonorcholesterol, this would mean that crosslinking to HMG-CoA reductase would occur only if the protein extended far enough into the lipid bilayer to lie in the vicinity of C-25 of the cholesterol side chain that contains the photosensitive azido group. Alternatively, if crosslinking could be demonstrated, then its ineffectiveness in changing reductase activity would only mean that the segment of HMG-CoA reductase involved in the crosslinkage was not critical for its activity.

The two alternatives could be resolved by the isolation of HMG-CoA reductase from these microsomal membranes, to demonstrate crosslinkage with azidonorcholesterol. Methods to solubilize HMG-CoA reductase as well as to isolate it from other contaminating proteins by gel electrophoresis have been developed (16), and the evidence should prove useful in describing the topography of this enzyme.

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