Effect of Bacteriohopane-32-ol on Lipid Metabolism in Hep G2 Cells

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To investigate the biological activity of the hopane group of pentacyclic triterpenes, the effect of bacteriohopane-32-ol (Monol) on lipid synthesis and secretion was determined using Hep G2 cells. Despite its structural similarity to 25-hydroxycholesterol, Monol did not affect free and esterified cholesterol synthesis determined by the incorporation from [^{14}C]acetate. Monol reduced the phospholipid secretion from Hep G2 cells without affecting cellular phospholipid synthesis from [^{3}H]glycerol. It also decreased the secretion of apolipoprotein B. These results suggest that the Monol-induced reduction in phospholipid secretion is due to a decrease in the number of lipoprotein particles secreted from Hep G2 cells.

Keywords hopanoid; oxysterol; Hep G2; lipid metabolism; apolipoprotein B

Hopanoids are pentacyclic triterpenoids that are widely distributed among prokaryotes. They have been found in strains of numerous microorganisms such as cyanobacteria, methylotrophs, purple non-sulphur bacteria and acetic acid bacteria.[1] These microorganisms contain a mixture of various hopanoids that differ from each other by the attachment of various polar side chains to the hydrophobic hopane core. Little is known about the function of these different compounds, except for the role of some hopanoids as membrane stabilizers.[2] Bacteriohopane-32-ol (Monol) (Fig. 1a) is a semi-artificial molecule derived from many common hopanepolyols, and has a molecular resemblance to cholesterol and 25-hydroxycholesterol (Fig. 1b).

Cholesterol is essential for the formation and function of the cellular membranes of mammals and is the obligate precursor of bile acids and steroid hormones.[3] Cholesterol is subject to oxidation by various active oxygen species, yielding biologically active oxysterols. Oxysterols are reported to influence such vital processes as *de novo* sterol biosynthesis, membrane function, deoxyribonucleic acid (DNA) synthesis, cell growth and proliferation, and aortal atherosclerosis associated with the parent cholesterol. It is well known that oxysterols such as 25-hydroxycholesterol reduce cholesterol synthesis in mammalian cells by decreasing the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase.[4] They also increase cholesterol esterification.[4] Thus, a concern naturally arises as to whether hopanoids are biologically active agents.

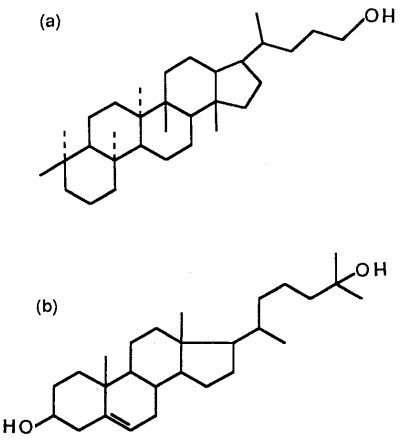

Fig. 1. Structure of Bacteriohopane-32-ol (a) and 25-Hydroxycholesterol (b)

In this study, we compared the effects of Monol on lipid synthesis and secretion in Hep G2 cells with those of 25-hydroxycholesterol.

Materials and Methods

Materials Monol was prepared as previously described.[5] 25-Hydroxycholesterol was purchased from Sigma (St. Louis, MO, U.S.A.). Both compounds were dissolved in ethanol and tested at a concentration of less than 0.2% ethanol (v/v). Under these conditions, ethanol had no significant effect on lipid synthesis and secretion. [2-^{14}C]Acetic acid sodium salt (51.6 mCi/mmol) and [^{3}H]glycerol (1 Ci/mmol) were obtained from Amersham International plc. (Buckinghamshire, England). All of the other chemicals were commercially available high purity materials.

Cell Cultures The established Hep G2 cell line, which was derived from human hepatoma, was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Cell stocks were grown in 80-cm^2 flasks containing medium A [Eagle's modified minimum essential medium (MEM, Flow Laboratories, McLean, VA, U.S.A.) supplemented with penicillin G (100 units/ml) and streptomycin (100 μg/ml)] with 10% (v/v) heat-inactivated fetal bovine serum (FBS), and were incubated in a humidified incubator (5% CO_2) at 37 °C.

Lipid Synthesis and Secretion The incorporation of [^{14}C]acetate and [^{3}H]glycerol into cellular and medium lipids was examined according to the method of Brown *et al.*,[6] with some modifications. On day 0, 1.2×10^5 cells were seeded in a 3.8-cm^2 plastic cell-well (Corning Glassworks, Corning, NY, U.S.A.) containing medium A with 10% FBS. On day 3 or 4, the medium was exchanged for fresh medium. On day 7, this medium was replaced with medium A. On day 8, the cells were preincubated with each compound in fresh medium A for 14 h and then labeled with 1 mM [^{14}C]acetate or 2.5 μM [^{3}H]glycerol for 4 h. At the end of the incubation, the plastic cell-wall was cooled in a cold bath, and the medium was harvested. The cells were washed three times with cold phosphate-buffered saline (PBS). Lipids in the cell homogenate or the culture medium were extracted by the method of Folch *et al.*[7] Individual lipids were separated by thin-layer chromatography as previously described.[8]

Enzyme-Linked Immunosorbent Assay (ELISA) The secreted apolipoprotein B (apo B) was assayed using a competitive ELISA method similar to that of Young *et al.*[9] The Hep G2 cells were treated under the same conditions as described in the section of the lipid synthesis and secretion. The cells were incubated with each compound in fresh medium A for 18 h and the medium was harvested. The 96-well microtiter plates (Falcon, NJ, U.S.A.) were coated with 0.2 ml of PBS containing 25 μg/ml of low density lipoprotein (LDL) as apo B for 18 h at 4 °C. The wells were washed with 0.2 ml of PBS containing 1% (w/v) bovine serum albumin (BSA) and 0.5% (v/v) Tween 20 (washing buffer). Residual binding sites on the plates were blocked by incubation with 0.2 ml of PBS containing 3% (w/v) BSA for 1 h at 23 °C. The wells were then washed three times with the washing buffer. Apo B was diluted with PBS containing 1% (w/v) BSA to provide LDL concentrations ranging from 0.05 to 0.8 μg/ml for the standard curve. Samples were diluted 4-fold in PBS containing 1% (w/v) BSA. Fifty μl of standards and samples were pipetted into the wells, followed immediately by 50 μl of a fixed concentration of monoclonal

antibody (MAB-012) (Chemicon International, Inc., U.S.A.) and apo B (diluted 30000-fold in PBS containing 3% BSA). The plates were incubated for 18 h at 4 °C. After washing three times with the washing buffer, 0.1 ml of horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 3000-fold in PBS containing 1% BSA) was added to each well. The plates were incubated at 23 °C for 1 h. After washing three times with the washing buffer, 0.1 ml of freshly prepared substrate solution [0.1 M citrate–phosphate buffer (32.3 mM citric acid, 35.4 mM $NaHPO_4$, pH 3.8) containing 0.02% of H_2O_2 and 1 mg/ml of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] was added and the plates were incubated for 30 min at 23 °C. The reaction was stopped by the addition of 50 μl of 0.2% NaN_3, and the absorbance in the wells was determined at 415 nm with a reader (MTP-100, Corona Electric Co., Ltd., Japan).

Determination of Protein Protein concentrations were determined according to the method of Lowry *et al.*[10] using BSA as a standard.

Data Analysis The data were statistically analyzed using Wilcoxon's rank-sum test.

Results and Discussion

Lipid Synthesis from Acetate and Glycerol The incorporation of [^{14}C]acetate into phospholipids, free cholesterol, free fatty acids, triglycerides and cholesteryl ester under the present experimental conditions was linear for up to at least 6 h of incubation (data not shown). As shown in Table I, Monol (20 μM) did not affect the synthesis of the lipids except for phospholipids. In contrast, 25-hydroxycholesterol (30 μM) reduced free cholesterol synthesis by 93% and increased cholesteryl ester synthesis by 38%. Oxysterol has been reported to reduce cholesterol synthesis by down-regulation of HMG-CoA reductase activity and enhance cholesteryl ester synthesis by increasing acyl CoA: cholesterol acyl transferase (ACAT) activity.[4] Monol did not affect free and esterified cholesterol synthesis from [^{14}C]acetate. Therefore, Monol is not considered to behave like oxysterol.

The incorporation of [^{13}H]glycerol into cellular phospholipids and triglycerides was linear for up to at least 4 h of incubation in Hep G2 cells (data not shown). As shown in Table I, Monol did not affect the synthesis of these two lipids from [^{13}H]glycerol.

Lipid Secretion from Glycerol Hep G2 cells secreate triglyceride-rich lipoprotein with the density of LDL, the apolipoprotein content of which is almost exclusively apo B.[11] To examine whether Monol affects lipid secretion from Hep G2 Cells, lipid secretion was determined using [^{3}H]glycerol as precursor. Figure 2 shows the effects of Monol and 25-hydroxycholesterol on the incorporation of [^{3}H]glycerol into lipids in the medium. Monol significantly reduced phospholipid secretion by 27% and 44% at 20 and 40 μM, respectively. However, whereas Monol tended to decrease triglyceride secretion (11% at 20 μM and 15% at 40 μM), the decreases were not significant. In addition, Monol at 40 μM did not affect cellular phospholipid synthesis (96% of control). 25-Hydroxycholesterol did not affect lipid secretion. These results indicate that Monol reduced phospholipid secretion from [^{3}H]glycerol in Hep G2 cells without affecting cellular phospholipid synthesis.

Secretion of Apolipoprotein B To examine whether the reduced phospholipid secretion induced by Monol resulted from a change in composition or a decrease in the number of particles secreted from Hep G2 cells, the secretion of apo B was determined. Apo B secretion into the medium was linear for up to at least 24 h of incubation (data not

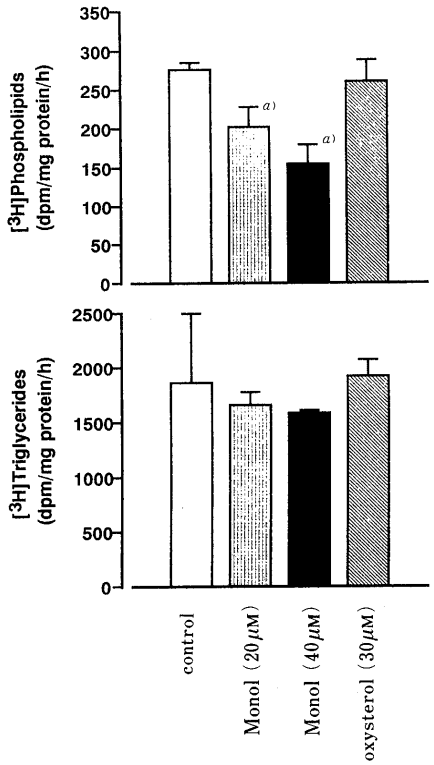

Fig. 2. Effects of Monol and Oxysterol on [^{3}H]Lipid Secretion from Hep G2 Cells Labeled with [^{3}H]Glycerol into the Culture Medium

On day 7, Hep G2 cells were preincubated with each compound for 14 h, after which 2.5 μCi/ml of [^{3}H]glycerol was added (final concentration; 2.5 μM). After incubation for 4 h, the phospholipids and triglycerides secreted into the culture medium were determined as described in Materials and Methods. Means ± S.D. are shown, $n=4$. *a)* Significantly different from controls, $p<0.05$.

TABLE I. Effects of Monol and Oxysterol on [^{14}C]Acetate and [^{3}H]Glycerol Incorporation into Lipids

Compounds	[^{14}C]Acetate incorporation ($\times 10^3$) (dpm/mg protein/h)					[^{3}H]Glycerol incorporation ($\times 10^3$) (dpm/mg protein/h)	
	PL	FC	FFA	TG	CE	PL	TG
Control	958 ± 109 (100)	514 ± 47 (100)	164 ± 20 (100)	820 ± 31 (100)	47 ± 3 (100)	64 ± 11 (100)	172 ± 10 (100)
Monol (20 μM)	1181 ± 58[a] (123)	551 ± 25 (107)	161 ± 3 (98)	826 ± 38 (101)	42 ± 2 (90)	75 ± 6 (117)	189 ± 16 (110)
Oxysterol (30 μM)	1184 ± 168 (124)	34 ± 4[a] (7)	150 ± 53 (92)	1232 ± 129 (109)	65 ± 4[a] (138)	53 ± 8 (82)	123 ± 12[a] (72)

On day 7, Hep G2 cells were preincubated with each compound for 14 h, after which [^{14}C]acetate (10 μCi/ml, 1 mM) or [^{3}H]glycerol (2.5 μCi/ml, 2.5 μM) was added. After incubation for 4 h, the phospholipids (PL), free cholesterol (FC), free fatty acids (FFA), triglycerides (TG) and cholesteryl ester (CE) in the cells were determined as described in Materials and Methods. Means ± S.D. are shown, $n=4$. Values in parentheses are percentages of control values. *a)* Significantly different from each control, $p<0.05$.

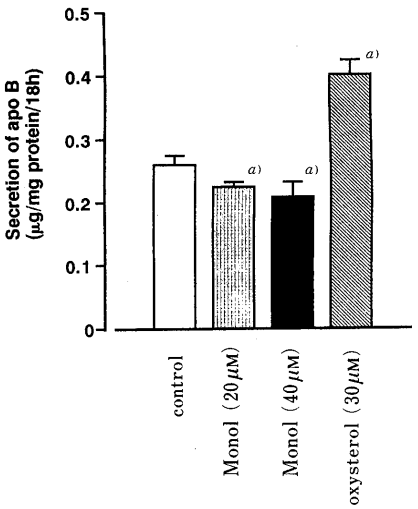

Fig. 3. Effects of Monol and Oxysterol on the Secretion of Apo B from Hep G2 Cells

On day 7, Hep G2 cells were incubated with each compound. After 18 h, the apo B from the Hep G2 cells was assayed as described in Materials and Methods. Means ± S.D. are shown, $n=4$. *a)* Significantly different from controls, $p<0.05$.

shown). As shown in Fig. 3, Monol decreased apo B secretion by 14% and 20% at 20 and 40 μM, respectively. These results suggest that the reduction in phospholipid secretion was due to a reduction in the number of lipoprotein particles secreted from the cells. 25-Hydroxycholesterol increased apo B secretion associated with an increase of cellular cholesteryl ester. Cianflone *et al.*[12] reported that an increase in cellular cholesteryl ester caused an increase in apo B secretion in Hep G2 cells. Therefore, accumulation of cholesteryl ester induced by 25-hydroxycholesterol may increase the secretion of apo B. Our data agree well with the results of Cianflone *et al.* Monol was found to decrease the secretion of apo B and lipids from Hep G2 cells without affecting lipid synthesis.

Pullinger *et al.*[13] reported that the apo B gene was constitutively expressed in Hep G2 cells and that the mechanism of acute regulation of apo B production by these cells must involve co- or post-translational processes. Maturation of lipoprotein particles occurs within the Golgi region.[14,15] The secretion of apo B may be regulated through its intracellular degradation, and a unique regulatory mechanism may exist for apo B secretion.[16] With respect to this point of view, Monol may be a useful reagent to investigate the maturational regulation and/or secretion of lipoprotein particles.

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