Effect of an Inhibitor of Squalene Epoxidase, NB-598, on Lipid Metabolism in Hep G2 Cells

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NB-598, a potent inhibitor of squalene epoxidase, inhibited cholesterol synthesis from [^{14}C]acetate and induced intracellular squalene accumulation in Hep G2 cells. NB-598 inhibited cholesterol synthesis from [^{14}C]acetate, [^{3}H]mevalonate, and [^{3}H]squalene, but not from [^{3}H]2,3-oxidosqualene in Hep G2 cells. It reduced cholesterol ester synthesis remarkably in the absence of exogenous cholesterol. This compound did not have any effect on the synthesis of ubiquinone and dolichol. When Hep G2 cells were prelabeled with micellar [^{3}H]cholesterol, NB-598 did not affect the excretion of bile acid incorporated from [^{3}H]cholesterol. However, NB-598 decreased the secretion of free and esterified cholesterol, triacylglycerol, and phospholipids, and increased the secretion of squalene. NB-598 is thought not only to inhibit cholesterol synthesis, but also to inhibit the secretion of lipids.

Keywords Hep G2 cell; cholesterol synthesis; NB-598; squalene epoxidase inhibitor; lipid secretion

Introduction

Squalene epoxidase (EC 1.14.99.7) is a microsomal enzyme that catalyzes the conversion of squalene into 2,3-oxidosqualene. Rat liver squalene epoxidase has been extensively studied by Bloch's group,[1-3] and it has been purified to homogeneity by Ono *et al.*[4,5] Squalene epoxidase appears to be regulated by the specific cholesterol pool in Hep G2 cells.[6] The activity of this enzyme is increased by the administration of hypocholesterolemic drugs such as cholestyramine and lovastatin and is decreased by the feeding of cholesterol in rats.[7,8] These results indicate that squalene epoxidase is an important regulatory enzyme in the cholesterol synthetic pathway.

NB-598 ((*E*)-*N*-Ethyl-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-3-[(3,3′-bithiophen-5-yl)methoxy]benzenemethanamine) was found by us to be a potent mammalian squalene epoxidase inhibitor.[9] NB-598 inhibited cholesterol synthesis from [^{14}C]acetate in rats, and it reduced serum cholesterol and triacylglycerol levels in dogs.[9,10]

A human hepatoma cell line, Hep G2, has recently been used to investigate lipid and lipoprotein metabolism.[11-13] In our previous experiments, we demonstrated the effects of simvastatin (MK-733, an HMG-CoA reductase inhibitor)[14] on lipid metabolism in Hep G2 cells[15] and found that the Hep G2 cell line is suitable for examining the effects of lipid-lowering agents *in vitro*. We therefore determined the effect of NB-598 on lipid metabolism using Hep G2 cells in the present study. A potent 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, L-654969 (the open acid form of simvastatin),[14] was used as a reference drug.

Materials and Methods

Materials NB-598 was synthesized in our laboratories. L-654969 was prepared by Merck Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). [2-^{14}C]Acetic acid, sodium salt (54.3 mCi/mmol), [9,10,(N)-^{3}H]oleic acid (5 Ci/mmol) and [2-^{3}H]glycerol (1 Ci/mmol) were obtained from Amersham International plc., Buckinghamshire, England. *RS*-[5-^{3}H(N)]Mevalonolactone (40 Ci/mmol), [^{3}H]acetic acid, sodium salt (86.4 mCi/mmol), [4,8,12,13,17,21,-^{3}H]squalene (26.9 Ci/mmol) and [1,2-^{3}H(N)]cholesterol (46.1 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]2,3-Oxidosqualene was prepared from *RS*-[5-^{3}H(N)]mevalonolactone with the aid of AMO-1618 (an inhibitor of 2,3-oxidosqualene lanosterol cyclase) according to the method of Knauss *et al.*[16] The radiopurity of these compounds was more than 97% when determined by thin-layer chromatography (TLC). Human lipoprotein-deficient serum (LPDS) was prepared as described by Brown *et al.*[17] Human low density lipoprotein (LDL, $d=1.020$—1.063) was isolated from freshly prepared plasma by preparative ultracentrifugation.[18] AMO-1618 was obtained from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals used were standard commercial high purity materials. Inhibitors were dissolved in dimethyl sulfoxide (DMSO). In all experiments, the concentration of DMSO did not exceed 0.3% (v/v). Under these conditions, DMSO had no significant effect on lipid synthesis.

Cell Cultures Hep G2[19] was obtained from ATCC (Rockville, MD, U.S.A.). Stock cultures of Hep G2 cells were maintained 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% heat-inactivated fetal bovine serum (FBS) and were incubated in a humidified incubator (5% CO_2) at 37 °C. For experiments, cells were seeded in 3.8-cm^2 plastic cell well (Corning Glassworks, Corning, NY, U.S.A.) or 9- and 24-cm^2 plastic petri dishes (Nunc, Roskilde, Denmark) at a density of 3×10^4 cells/cm^2 with medium A containing 10% FBS. The medium was changed every 2—3 d. After the cell layer became nearly confluent (5—6 d after plating), all of the studies described were started.

Cholesterol and Squalene Synthesis from Various Precursors in Hep G2 Cells Medium was replaced with medium A containing 10% LPDS on the day before the experiments. Nearly confluent Hep G2 cells in 9-cm^2 dishes were preincubated with each inhibitor in fresh medium A (1 ml) containing 10% LPDS for 1 h and then labeled with [^{14}C]acetate, [^{3}H]mevalonate, [^{3}H]squalene and [^{3}H]2,3-oxidosqualene for 6 h. [^{14}C]Acetate and [^{3}H]mevalonolactone were added to the dishes as aqueous solutions while the other two precursors were added as ethanolic solutions. After washing with cold phosphate-buffered saline (PBS) three times, the cells were scraped with a rubber policeman. Cellular lipids were extracted, saponified with ethanolic KOH and separated by TLC (silica gel TLC, Merck, Art. 5583) using hexane–diethyl ether–acetic acid (85 : 15 : 4, v/v) as a solvent system.[15] Authentic standards on TLC were visualized by exposure to I_2 vapor. The distribution of radioactivity on TLC was also detected using a radiochromatoscanner (LB-282, Berthold, Wildbad, West Germany), and the radioactivity in the area corresponding to authentic standard was counted in a liquid scintillation counter.

Esterification of Cholesterol from [^{3}H]Oleate Hep G2 cells in 9-cm^2 dishes were incubated in fresh medium A (1 ml) containing 10% LPDS with or without 100 μg/ml of LDL for 24 h. After 1 h of preincubation with or without inhibitor, the cells were labeled with 0.1 mM [^{3}H]oleate bound to bovine serum albumin (BSA, fatty acid free) for 1 h.[20] The cells were then washed with cold PBS three times and harvested. The lipids in the cells were extracted by the method of Folch *et al.*[21] The cholesteryl [^{3}H]oleate was separated on silica gel TLC plates using hexane–diethyl ether–acetic acid (85 : 15 : 4, v/v) as a solvent system and the radioactivity in the spot corresponding to authentic cholesteryl oleate was counted. Esterification activity is expressed as pico-mol of cholesteryl [^{3}H]oleate formed per mg of cell protein per h.

Ubiquinone and Dolichol Synthesis The synthesis of ubiquinone and dolichol was determined as previously described.[22] Hep G2 cells in 24-cm^2 dishes containing fresh medium A supplemented with 10% LPDS

(3 ml) were incubated with each inhibitor and [^{3}H]acetate for 18 h. After washing with PBS, cellular lipids were extracted by the method of Folch *et al.*[21] Ubiquinone and dolichol were separated by two-dimensional TLC (Merck, Art. 5554) according to the method of Sexton *et al.*[23] Ubiquinone (coenzyme Q10) and dolichol (C_{80}—C_{105}) were used as markers. Authentic standards on TLC were visualized by exposure to I_2 vapor. The bands corresponding to authentic cholesterol, ubiquinone and dolichol were scraped and their radioactivity was counted by scintillation counter.

Lipid and Bile Acid Secretion For the determination of lipid secretion into the medium, the medium was replaced with serum-free medium A on the day before the experiments. Nearly confluent cells in 3.8 cm^2 dishes were pretreated with each inhibitor in serum free medium A (1 ml) for 18 h. Four hours before the end of incubation, [^{14}C]acetate or [^{3}H]glycerol was added to the medium. After incubation, the secreted lipid in the medium was extracted by the method of Folch *et al.*[21] When [^{3}H]glycerol was used as precursor, the chloroform phase was extensively washed with 0.04 N HCl–methanol (1 : 1, v/v) to remove excess [^{3}H]glycerol. Various lipid classes were separated on a silica gel G plate using hexane–diethyl ether–methanol–acetic acid (85 : 15 : 1 : 1, v/v) as a solvent system for phospholipids, free cholesterol and triacylglycerol. Another solvent system (petroleum ether : diethylether : acetic acid = 95 : 5 : 0.5, v/v) was also used for esterified cholesterol and squalene separation. Radioactivity of the lipids was quantified as described in the section on cholesterol and squalene synthesis from various precursors. Cholesterol, cholesterol oleate, triolein, phosphatidylcholine dipalmitoyl and squalene were used as authentic standards.

For the determination of the secretion of bile acids, cholesterol and cholesterol ester, cells in 3.8-cm^2 dishes were prelabeled with micellar [^{3}H]cholesterol (cholesterol : sodium oleate : sodium taurocholate = 20 μM : 200 μM : 160 μM) in the presence of medium A (1 ml) containing 1% BSA (essentially fatty acid free) for 9 h. The cell layer was washed with the same medium twice and extensively washed with serum-free medium A twice to completely remove excess micellar [^{3}H]cholesterol from the cells. After washing, cells were incubated in serum-free medium A (1 ml) with each inhibitor for 18 h. To determine the bile acid secretion, the medium was acidified with 1 N HCl and the secreted bile acids were extracted with chloroform–methanol (2 : 1, v/v)[21] and the solvent layer was re-extracted with 0.5 N NaOH. This water layer was used as the bile acid fraction. For the determination of cholesterol and cholesterol ester excretion, the substances were extracted from the medium, separated on TLC and quantified as described above.

Determination of Protein Protein concentrations were determined according to the method of Lowry *et al.*[24] using BSA as a standard.

Data Analysis Data from these studies were statistically analyzed using Wilcoxon's rank-sum test. Variations in all mean values in the tables and figures are expressed as standard deviations (S.D.).

Results

Cholesterol Synthesis in Hep G2 Cells Cholesterol synthesis from [^{14}C]acetate was linear for up to at least 6 h of incubation in Hep G2 cells (data not shown). Hep G2 cells were incubated in the presence of NB-598 or L-654969. Figure 1 shows the distribution of radioactivity of non-saponifiable lipids on TLC. L-654969 (100 nM) inhibited cholesterol synthesis but did not cause accumulation of squalene in Hep G2 cells. NB-598 (100 nM) inhibited cholesterol synthesis and induced accumulation of intracellular squalene. One hundred nM NB-598 inhibited cholesterol synthesis by 96% and the same concentration of L-654969 inhibited it by 78%. IC_{50} values of NB-598 and L-654969 for cholesterol synthesis were 3.4 and 6.6 nM, respectively.

Cholesterol Synthesis from Various Precursors in Hep G2 Cells To investigate the inhibitory specificity of NB-598 on several cholesterol synthetic pathway-enzymes in Hep G2 cells, the effect of NB-598 on cholesterol synthesis from various precursors was examined. Results are summarized in Table I. L-654969 (100 nM) inhibited cholesterol synthesis from [^{14}C]acetate, but not from [^{3}H]mevalonate, [^{3}H]squalene or [^{3}H]2,3-oxidosqualene. On the other hand, NB-598 (100 nM) inhibited cholesterol synthesis from [^{14}C]acetate, [^{3}H]mevalonate, and [^{3}H]squalene and increased the intracellular level of squalene. NB-598 did not inhibit cholesterol synthesis from [^{3}H]2,3-oxidosqualene. These results show that L-654969 is a specific HMG-CoA reductase inhibitor and NB-598 is a specific squalene epoxidase inhibitor.

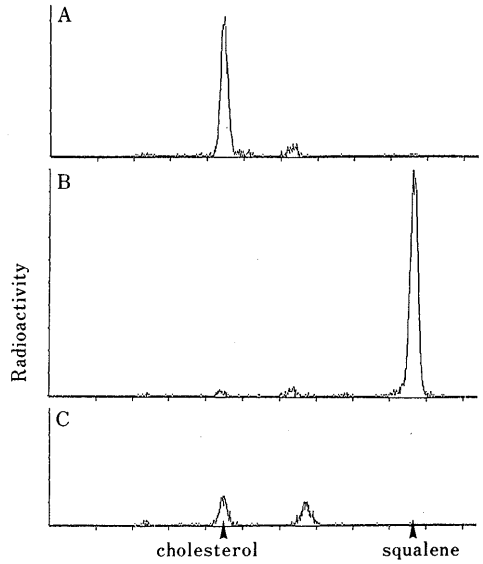

Fig. 1. The Distribution of Radioactivity of Non-saponifiable Lipids on TLC Produced by Treatment with NB-598 or L-654969 in Hep G2 Cells

Cells in 1 ml of medium A supplemented with 10% LPDS were preincubated with each inhibitor for 1 h; 1 μCi of [^{14}C]acetate (final concentration 1 mM) was then added. After 6 h of incubation, the cells were saponified and the non-saponifiable lipids were extracted and separated on TLC as described in Materials and Methods. A) control, B) 100 nM NB-598, C) 100 nM L-654969. The radioactivity on TLC plate was detected with a radiochromatoscanner.

TABLE I. Effects of NB-598 and L-654969 on Cholesterol Synthesis from Various Precursors and Accumulation of Squalene in Hep G2 Cells

Precursor	Compound	(nM)	dpm/mg cell protein/h	
			Cholesterol	Squalene
[2-^{14}C]Acetate		(0)	8760±1130	144±7
	NB-598	(100)	380±45[a]	24500±2370[a]
	L-654969	(100)	1960±152[a]	66±7[a]
[5-^{3}H]Mevalonate		(0)	3150±285	60±7
	NB-598	(100)	76±3[a]	3680±113[a]
	L-654969	(100)	2858±487	108±8[a]
[^{3}H]Squalene		(0)	8120±615	—
	NB-598	(100)	576±40[a]	—
	L-654969	(100)	9220±977	—
[^{3}H]2,3-Oxidosqualene		(0)	40000±644	—
	NB-598	(100)	44300±6030	—
	L-654969	(100)	42800±3890[a]	—

Cells were preincubated with NB-598 or L-654969 for 1 h; one of the following precursors, [^{14}C]acetate (1 μCi/ml, 1 mM), [^{3}H]mevalonolactone (1 μCi/ml, 50 μM), [^{3}H]squalene (1 μCi/ml, 5 μM) or [^{3}H]2,3-oxidosqualene (0.5 μCi/ml) was then added to the culture medium. After 6 h of incubation, the cells were saponified. Procedures to determine the radioactivity of cholesterol and squalene are shown in Materials and Methods. Each value is the mean±S.D. of triplicate determinations. Significantly different from the value of each control, *a*) $p \leq 0.05$; —, not tested.

Esterification of Cholesterol from [^{3}H]Oleate in Hep G2 Cells [^{3}H]Oleate was incorporated into cholesteryl [^{3}H]oleate in a linear fashion after up to 3 h of incubation in the absence or presence of LDL (data not shown). As

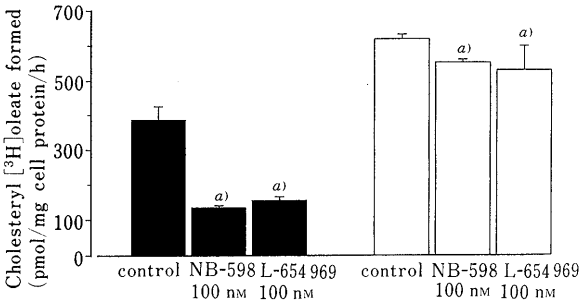

Fig. 2. Effects of NB-598 and L-654969 on Cholesterol Ester Synthesis in Hep G2 Cells

Before the experiment, cells in 1 ml of medium A containing 10% LPDS were incubated with or without 100 μg/ml of LDL for 24 h. The cells were preincubated with NB-598 or L-654969 for 1 h and then labeled with 2 μCi of [^{3}H]oleate bound to albumin (final concentration 0.1 mM) for 1 h. The synthesized cholesteryl [^{3}H]oleate was extracted, separated and quantified as described in Materials and Methods. Each value represents the mean $\pm$ S.D. of triplicate determinations. ■, $-$LDL; □, $+$LDL. Significantly different from the value in each control, *a*) $p \leq 0.05$.

Table II. Effects of NB-598 on the Incorporation of [^{3}H]Acetate into Ubiquinone 10, Dolichol and Cholesterol in Hep G2 Cells

	Incorporation of [^{3}H]acetate into end products		
	Ubiquinone 10	Dolichol	Cholesterol
	(dpm/mg cell protein/18 h)		
Control	1970 $\pm$ 248	813 $\pm$ 176	568000 $\pm$ 11700
NB-598 1 μM	2487 $\pm$ 713	782 $\pm$ 99	24420 $\pm$ 2420*a)*

Hep G2 cells in 3 ml of fresh medium A containing 10% LPDS were incubated with NB-598 and [^{3}H]acetate (3 mM, 15 μCi/ml). After 18 h of incubation, ubiquinone, dolichol and cholesterol were extracted and the radioactivity of each fraction was quantified as described in Materials and Methods. Each value represents the mean $\pm$ S.D. of triplicate determinations. Significantly different from the value in each control, *a*) $p \leq 0.05$.

Table III. Effect of NB-598 on Bile Acid Secretion

		(dpm/mg cell protein/18 h)
Control		113600 $\pm$ 8368
NB-598	1 μM	100600 $\pm$ 8109
L-694969	1 μM	105300 $\pm$ 8148

To determine the secretion of bile acids, cells were prelabeled with micellar [^{3}H]cholesterol (cholesterol : sodium oleate : sodium taurocholate = 20 μM : 200 μM : 160 μM) in the presence of medium A (1 ml) containing 1% BSA for 9 h. After washing, cells were incubated in serum free medium A (1 ml) with each inhibitor for 18 h. The medium was acidified with 1 N HCl, the secreted bile acids were extracted with chloroform–methanol (2 : 1, v/v) and the solvent layer was re-extracted with 0.5 N NaOH. The radioactivity of this water layer was considered the bile acid fraction. Each value represents the average $\pm$ S.D. of 4 experiments.

shown in Fig. 2, LDL increased the incorporation of [^{3}H]oleate into cholesteryl ester by 1.6 times. NB-598 (100 nM) and L-654969 (100 nM) decreased the incorporation of [^{3}H]oleate into cholesteryl [^{3}H]oleate in the presence or absence of LDL. However, the inhibitory effect of NB-598 was far weaker in the presence than in the absence of LDL. Similar effects have also been observed with L-654969 as described in a previous report.[15] Cholesteryl ester synthesis by these inhibitors therefore appears to be due to the inhibition of cholesterol synthesis. NB-598 inhibited cholesteryl ester synthesis as potently as did L-654969.

Ubiqinone and Dolichol Synthesis in Hep G2 Cells To investigate the effects of NB-598 on ubiquinone and dolichol synthesis in Hep G2 cells, [^{3}H]acetate was used

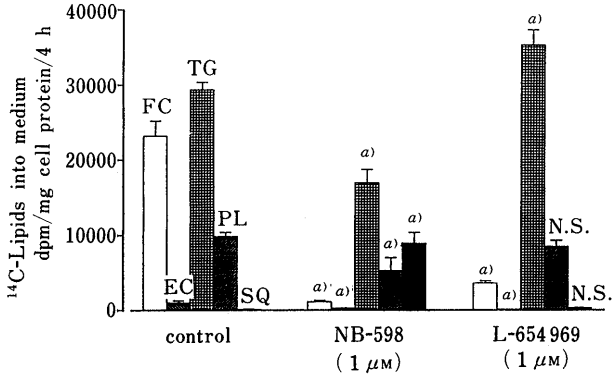

Fig. 3. Effect of NB-598 on the Lipid Secretion Incorporated from [^{14}C]Acetate into Medium

Cells were incubated in serum free medium A (1 ml) with each inhibitor (1 μM) for 18 h. Four hours before the end of incubation, 10 μCi of [^{14}C]acetate (final concentration 1 mM) was added to the medium. Secreted lipids in the medium were extracted and separated on TLC as described in Materials and Methods. Abbreviations are as follows: FC: free cholesterol, EC: esterified cholesterol, TG: triacylglycerol, PL: phospholipids, SQ: squalene. Significantly different from the value in the control, *a*) $p < 0.05$. N.S.: not significantly different from control.

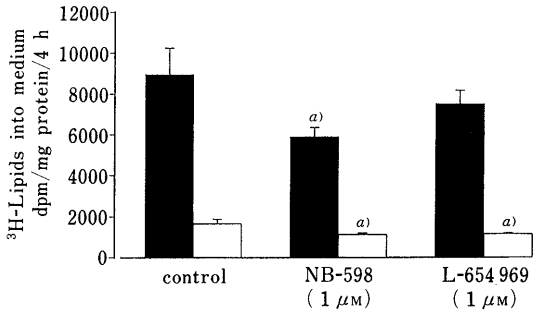

Fig. 4. Effect of NB-598 on Lipid Secretion Incorporated from [^{3}H]Glycerol into Medium

Cells were incubated in serum free medium A (1 ml) with each inhibitor for 18 h. Four hours before the end of incubation, 2.5 μCi of [^{3}H]glycerol (carrier free) was added to the medium. Secreted lipids in the medium were extracted and separated on TLC as described in Materials and Methods. Abbreviations are as follows: ■, triacylglycerol; □, phospholipids. Significantly different from the value in the control, *a*) $p < 0.05$.

as a precursor for the synthesis of the isoprenoid chain of ubiquinone and dolichol. Table II shows the effects of NB-598 on the incorporation of [^{3}H]acetate into cholesterol, ubiquinone and dolichol. At a concentration of 1 μM, NB-598 markedly inhibited cholesterol synthesis (96% inhibition) but did not affect ubiquinone or dolichol synthesis.

The Effect of NB-598 on Bile Acid Secretion Hep G2 cells were labeled by treatment with micellar [^{3}H]-cholesterol and the effects of NB-598 on bile acid secretion in the medium were examined. As shown in Table III, neither NB-598 nor L-654969 affected bile acid secretion from cells.

The Effect of NB-598 on Lipid Secretion The 18 h-incubation with 1 μM NB-598 or L-654969, the incorporation of [^{14}C]acetate into secreted lipids was examined for the last 4 h. Labeled lipids from [^{14}C]acetate were predominant in the lipoprotein fraction ($d < 1.063$) (data not shown). Therefore, the radioactivity in each lipid fraction in the medium was used to indicate secreted lipoprotein lipids. As shown in Fig. 3, NB-598 and L-654969 both dramatically reduced the secretion of radioactivity in free and esterified cholesterol. NB-598 reduced

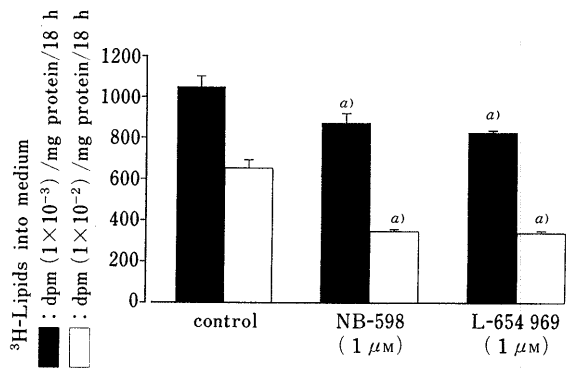

Fig. 5. Effect of NB-598 on Cholesterol and Cholesterol Ester Secretion into Medium

Cells were prelabeled with [^{3}H]cholesterol by treatment with micellar [^{3}H]cholesterol. Prelabeled cells in serum free medium A (1 ml) were incubated with each inhibitor. After 18 h-incubation, secreted lipids in the medium were extracted and separated on TLC as described in Materials and Methods. Abbreviations are as follows: ■, free cholesterol; □, esterified cholesterol. Significantly different from the value in the control, *a*) $p<0.05$.

the radioactivity in triacylglycerol by 43%, but L-654969 increased it by 20%. NB-598 and L-654969 decreased the radioactivity in phospholipids by 47 and 14%, respectively. NB-598 increased the radioactivity in squalene by approximately 110 fold, but L-654969 did not affect it. We also examined the effect on the incorporation of [^{3}H]glycerol into triacylglycerol and phospholipids to examine the isotope dilution effect under the same experimental conditions. As shown in Fig. 4, NB-598 and L-654969 both reduced the incorporation of radioactivity into phospholipids by 32 and 30%, respectively. NB-598 decreased the radioactivity of triacylglycerol by 34% and L-654969 also decreased it, but not significantly. We also examined the direct effect of NB-598 on the secretion of cholesterol. Cells were labeled with micellar [^{3}H]-cholesterol and the excreted free and esterified cholesterol was examined. NB-598 and L-654969 both decreased free and esterified cholesterol into the medium (Fig. 5).

Discussion

Hepatocytes synthesize lipids such as sterols, triacylglycerol and phospholipids. The synthesis and secretion of hepatic lipids and lipoproteins are exquisitely regulated by considerable number of factors (hormones, nutrients, metabolites, *etc.*). Hepatocytes also take up the lipids carried by lipoproteins through LDL receptors. Cohen *et al.*[13] reported that a human hepatoma cell line, Hep G2, was suitable for examining lipid metabolism. We demonstrated that this cell line was also appropriate for determining the effect of a hypolipidemic agent.[15]

NB-598 and L-654969 inhibited cholesterol synthesis from [^{14}C]acetate in Hep G2 cells in a dose-dependent manner.[9,15] NB-598 increased and L-654969 decreased cellular squalene levels.

NB-598 inhibited cholesterol synthesis from [^{14}C]-acetate, [^{3}H]mevalonate and [^{3}H]squalene, but not from [^{3}H]2,3-oxidosqualene. These results show that NB-598 is a specific squalene epoxidase inhibitor of the cholesterol synthetic pathway in human cells. L-654969 inhibited cholesterol synthesis from [^{14}C]acetate, but not from [^{3}H]mevalonate, [^{3}H]squalene or [^{3}H]2,3-oxidosqualene; therefore, L-654969 acts as a specific HMG-CoA reductase inhibitor in human cells. The incorporation of [^{14}C]-acetate into squalene was 2.8 times higher in the presence of NB-598 (100 nM) than that of [^{14}C]acetate into cholesterol in the absence of the inhibitor (Table I). However, the incorporation of [^{3}H]mevalonolactone to squalene in cells treated with NB-598 was similar to that of [^{3}H]mevalonolactone to cholesterol in non-treated cells. We reported[25] that HMG-CoA reductase was induced in Hep G2 cells following depletion of the cholesterol pool by NB-598. These data indicate that the HMG-CoA reductase activity induced by NB-598 increased the incorporation of [^{14}C]acetate into squalene. NB-598 and L-654969 inhibited cholesterol ester synthesis from [^{3}H]-oleate in Hep G2 cells because these compounds reduced cholesterol synthesis, which is the substrate of acyl-CoA: cholesterol acyltransferase (ACAT).[26] The inhibitory activity of these compounds was weaker in the presence than in the absence of LDL, since cholesterol was supplied from LDL in the medium.

The isoprenoid chain of ubiquinone and dolichol is derived from mevalonate, which also serves as a precursor for sterol synthesis in eucaryotic cells. Maltese and Aprille[27] showed that mevinolin (lovastatin), a competitve inhibitor of HMG-CoA reductase, inhibited ubiquinone synthesis incorporated from [^{3}H]acetate in cultured neuroblastoma cells. Keller[28] showed that mevinolin also inhibited the synthesis of dolichoylphosphate in the same manner as did cholesterol in rat liver slices, since HMG-CoA reductase inhibitors decreased mevalonate production by inhibiting HMG-CoA reductase. Squalene epoxidase is located in the middle of the cholesterol synthetic pathway, past the branch points for ubiquinone and dolichol biosynthesis. The effects of NB-598 on ubiquinone and dolichol biosynthesis in Hep G2 cells were therefore studied using [^{3}H]acetate as a precursor. As expected, NB-598 inhibited neither ubiquinone nor dolichol synthesis in Hep G2 cells because it did not inhibit the isoprenoid chain supply. These results indicate that NB-598 is a specific mammalian squalene epoxidase inhibitor.

NB-598 increased the secretion of squalene into medium, but L-654969 did not affect it. In a dog experiment,[9,10] serum squalene levels were increased by the treatment with NB-598; therefore, one of the origins of serum squalene is thought to be the liver. NB-598 and L-654969 both decreased the secretion of cholesterol and phospholipids. NB-598 decreased triacylglycerol secretion, but L-654969 did not affect it. NB-598 and L-654969 decreased the secretion of cholesterol and cholesterol ester from Hep G2 cells labeled with micellar [^{3}H]cholesterol. The intracellular cholesterol pool is thought to be decreased by the treatment with NB-598 or L-654969, because these compounds inhibit the synthesis of cholesterol potently. If these inhibitors do not affect the secretion of cholesterol, the radioactivity of cholesterol in the medium would be expected to increase because of the isotope concentration effect. However, the radioactivity of cholesterol into the medium was found to be decreased by the treatment of these compounds. Therefore, NB-598 and L-654969 are thought not only to inhibit cholesterol synthesis, but also to inhibit the secretion of cholesterol. Cianflone *et al.*[29] reported that lovastatin (HMG-CoA reductase inhibitor) decreased the secretion of triacylglycerol and cholesterol

ester from Hep G2 cells into the medium. Sato *et al.*[30] also reported that Hep G2 cells secreted LDL- and HDL-like lipoprotein which were poor in cholesterol by the treatment of CS-514, another HMG-CoA reductase inhibitor. In our previous experiment,[10] we found that NB-598 strikingly decreased serum cholesterol and triacylglycerol levels. NB-598 did not affect the triacylglycerol synthesis.[9] To decrease serum levels of cholesterol and triacylglycerol, inhibition of VLDL secretion or increase of VLDL degradation would be necessary. We have already found that NB-598 increased LDL receptor activity in Hep G2 cells.[25] The inhibitory effect of NB-598 on lipid secretion observed in cells may contribute to the lipid lowering effects of NB-598 in addition to the increase of LDL receptor activity. We will examine the exact mechanism of the inhibitory effect of NB-598 on lipoprotein secretion in a following paper.

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