EFFECT OF NATURAL AND SYNTHETIC ANTIOXIDANTS (POLYHYDROXYNAPHTHAQUINONES) ON CHOLESTEROL METABOLISM IN CULTURED RABBIT HEPATOCYTES

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Antioxidants are nowadays regarded as promising antiatherogenic and hypolipidemic agents. It has been shown that such familiar antioxidants as α-tocopherol and also the substance probucol, which has proved itself already in clinical practice, possess a hypocholesterolemic action [4, 15]. It has recently been shown that polyhydroxynaphthaquinones (PHNQ), which include natural pigments contained in the shells and spines of sea urchins, possess high antioxidant activity [2]. In a search for new hypolipidemic and antiatherogenic preparations, in the investigation described below the effect of PHNQ was studied on certain parameters of cholesterol metabolism in hepatocytes in vitro. The comparison preparation was the widely known antioxidant α-tocopherol. Choice of rabbit hepatocytes in culture as the model system for testing these compounds was based on the following factors. First, the liver, or more precisely its principal cells, the hepatocytes, plays a leading role in the regulation of the plasma cholesterol level. Second, it has already been established that antioxidants such as α-tocopherol and probucol realize their hypolipidemic effect by modifying cholesterol metabolism actually in the hepatocytes. Third, under primary culture conditions hepatocytes preserve the properties which they possess in vivo for several days.

EXPERIMENTAL METHOD

PHNQ were obtained from sea urchin shells by the method in [1]. The following substances were used: α-tocopherol (from "Sigma," USA), sodium [2-^{14}C] acetate (specific activity 40-60 mCi/mmole) and [4-^{14}C] cholesterol (specific activity 50-60 mCi/mmole) were obtained from "Amersham International," UK. Hepatocytes were isolated from male Chinchilla rabbit livers by perfusion of the organ with Eagle's medium containing 0.1% collagenase [1]. The cells were resuspended in Eagle's minimal medium containing 10% fetal calf serum (FCS) and seeded in a density of $2 \cdot 10^5/cm^2$ in plastic Petri dishes. The dishes were kept in an incubator at 37°C in an atmosphere of 95% air and 5% CO_2. High-density lipoproteins of the second fraction (HDL_2) were obtained from healthy human plasma by ultracentrifugation [11]. HDL_2 labeled with [4-^{14}C] cholesterol, were obtained by the method described previously [13]. HDL_2 labeled with [4-^{14}C] cholesterol with specific activity of $1.2 \cdot 10^7$ cpm/mg protein were used in the experiments. Isolation of neutral lipids from the cells and of bile acids was carried out by differential extraction with a chloroform:methanol mixture (1:1 by volume), as described previously [9]. Individual classes of neutral lipids were fractionated by high-performance thin-layer chromatography. Incorporation of the radioactive label into cholesterol

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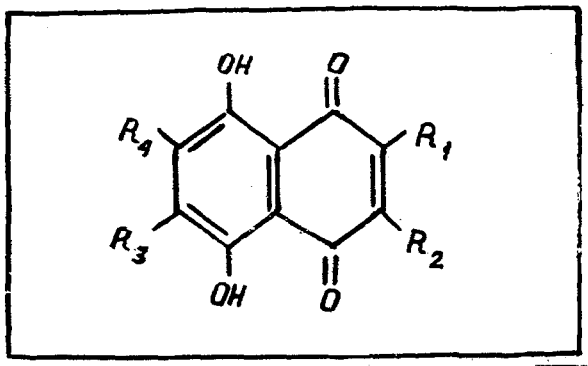
TABLE 1. Effect of PHNQ on Synthesis of [^{14}C] Cholesterol in Cultured Hepatocytes (M ± SD)



Antioxidant, μM		[^{14}C] cholesterol, percent of control	Substituents in naphthazarine molecule
Echinochrome	10	92,5±5,0	$R_1=R_2=R_4=OH$,
	100	85,6±5,6	$R_3=C_2H_5$
Histochrome	10	96,9±6,4	$R_1=R_2=ONa$,
	100	75,2±5,8*	$R_3=C_2H_5$, $R_4=OH$
A400	10	86,0±7,0	$R_1=R_2=R_4=OH$,
	100	81,3±5,7	$R_3=CH_3$
A572	10	95,5±9,1	$R_1=R_2=GSH$,
	100	73,8±7,6*	$R_3=C_2H_5$, $R_4=OH$
A657	10	97,0±8,1	$R_1=R_2=GSH$,
	100	89,0±6,8	$R_3=OH$, $R_4=CH_3$
A658	10	95,6±7,3	$R_1=R_2=GSH$,
	100	89,8±6,1	$R_3=R_4=OH$
	10	113,3±4,4	
α-Tocopherol	100	145,7±10,4*	

Legend. *$p < 0.05$: significance of differences from control; GSH) glutathione.

and total bile acids was determined by scintillation radiometry. Bile acids were determined quantitatively by densitometry after their preliminary fractionation by thin-layer chromatography [7]. Protein of cells and lipoproteins was determined by Lowry's method [8]. The results were subjected to statistical analysis by Student's t test. The experimental data are presented as mean values ± standard deviation (n = 3).

EXPERIMENTAL RESULTS

Table 1 gives data on the effect of some PHNQ and α-tocopherol on the rate of cholesterol synthesis. To determine it, hepatocytes were preincubated for 24 h in medium containing 10% FCS in the presence of the concentrations of antioxidant indicated, after which the medium was replaced by serum-free medium with the addition of 5 μCi [^{14}C] acetate and incubated for 3 h. Incorporation of the label into the cell cholesterol was then determined. It was found that cholesterol synthesis was significantly inhibited by only two compounds: histochrome and its analog A572 by 20-25% and 25-30% respectively (Table 1). Conversely, α-tocopherol increased incorporation of [^{14}C] acetate into cholesterol by 35-55%, indicating stimulation of cholesterol synthesis.

To determine the rate of synthesis of bile acids from [4-^{14}C] cholesterol, 1 μCi of [4-^{14}C] cholesterol, diluted in 5 μl of FCS, was added to standard culture medium and incubated with the cells for 48 h. The results given in Fig. 1 show that both histochrome and α-tocopherol, in a concentration of 10-100 μM cause dose-dependent stimulation of bile acid synthesis. The other preparations of PHNQ studied did not affect bile acid synthesis (results not given).

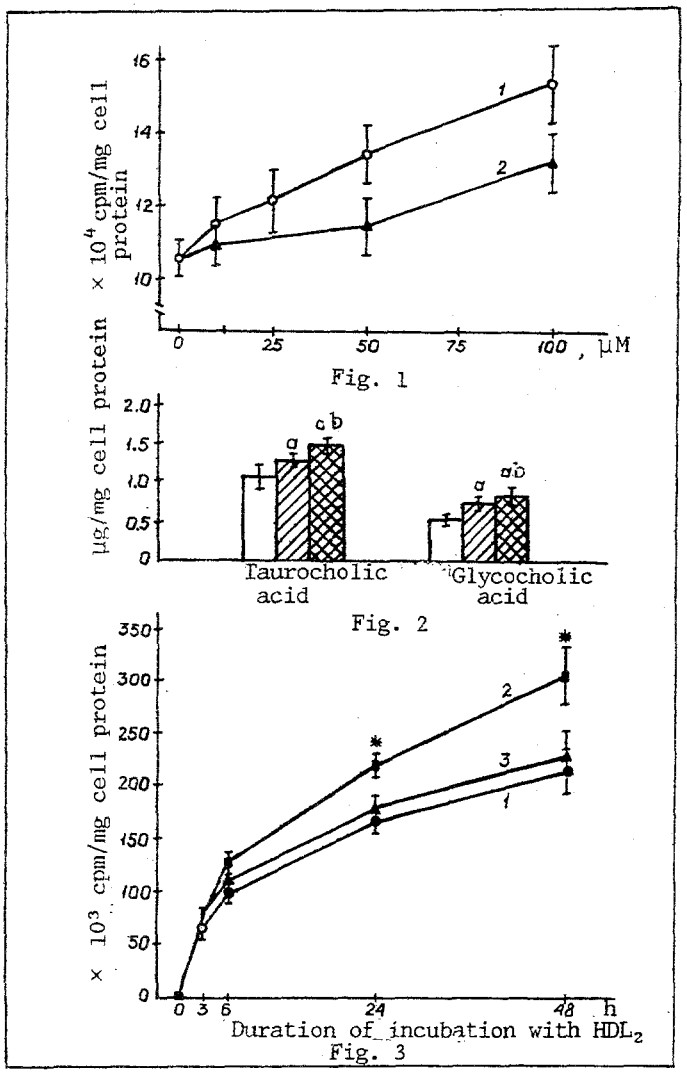


Fig. 1. Effect of histochrome and α-tocopherol on synthesis of [^{14}C] bile acids by cultured hepatocytes. Values given in the form M ± m; 1) histochrome, 2) tocopherol.

Fig. 2. Effect of HDL_2 and histochrome on secretion of conjugated bile acids by cultured hepatocytes. Unshaded columns – control, obliquely shaded – HDL_2 250 μg/ml; cross-hatched – HDL_2 + 100 μM histochrome. Values given in the form M ± m; a) $p < 0.05$: significance of differences compared with control; AB) $p < 0.05$: significance of differences compared with HDL_2.

Fig. 3. Dynamics of accumulation of [^{14}C] cholesterol of HDL_2 in cultured hepatocytes. 1) Control, 2) 100 μM histochrome; 3) 100 μM α-tocopherol. Values given in the form M ± m; *$p < 0.05$: significance of differences compared with control.

Previous investigations on liver cell cultures [5, 9] showed that an increase in the cholesterol concentration in hepatocytes is accompanied by stimulation of the rate of bile acid synthesis. Direct proof has recently been obtained that bile acid production may be regulated directly by cholesterol, the substrate for the key enzyme of bile acid biosynthesis, 7α-hydroxylase [14]. The form, or in other words the composition of the class of lipoproteins in which cholesterol is supplied to the hepatocyte, determines the efficiency of its use in the cells as substrate for 7α-hydroxylase [9]. The main source of substrate for 7α-hydroxylase has been shown to be cholesterol, supplied to hepatocytes in the composition of HDL_2, and synthesized de novo [3, 6]. On the basis of data in the literature and our own results it can be postulated that stimulation of bile acid synthesis by α-tocopherol is the result of activation of cholesterol synthesis. Since histochrome inhibits cholesterol synthesis, stimulation of bile acid production by this substance may be based on an increase in the flow of cholesterol into the cell in the composition of HDL_2.

It follows from the results in Fig. 2 that incubation of hepatocytes with HDL_2 (250 μg protein/ml) for 48 h led to stimulation of synthesis of conjugated bile acids by 20-25%. In the presence of histochrome (100 μM) and HDL_2 a further increase in synthesis and secretion of bile acids was observed.

The dynamics of accumulation of [4-^{14}C] cholesterol of HDL_2 in hepatocytes in the presence and absence of antioxidants is shown in Fig. 3. For this purpose the cells were cultured in serum-free medium with the addition of [4-^{14}C] cholesterol-labeled HDL_2 (250 μg protein/ml). After 3, 6, 24, and 48 h, the concentration of [4-^{14}C] cholesterol of HDL_2 in the hepatocytes was recorded on a scintillation counter. Unlike α-tocopherol, histochrome increased the intracellular content of [^{14}C] cholesterol of HDL_2. These findings confirm our hypothesis that histochrome stimulates bile acid synthesis directly through an increase in the supply of cholesterol to hepatocytes in the composition of HDL_2.

The experiments thus showed that histochrome stimulates bile acid production in the presence of serum cholesterol or HDL_2 in the culture medium. We know that the predisposition for the development of hypercholesterolemia and atherosclerosis is enhanced in people with a low level of bile acid synthesis [10]. The results of the present investigation are evidence that histochrome inhibits cholesterol synthesis and stimulates bile acid synthesis by cultured rabbit hepatocytes, and that the effect of histochrome is comparable with that of α-tocopherol, suggesting that this substance possesses hypocholesterolemic activity.

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