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Effects of khellin and timefurone on secretion and catabolism of lipoproteins by cultured rabbit and human hepatocytes

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Summary

The effects of timefurone and khellin on lipoprotein metabolism in cultured rabbit and human hepatocytes were characterized by the following parameters: (1) the uptake and degradation of ^{125}I-LDL; (2) secretion of total apolipoprotein B (apo-B) as measured by enzyme-linked immunosorbent assay (ELISA); (3) VLDL [^{35}S]methionine labeled apo-B and apo-E secretion; and (4) cholesterol (CH) synthesis and CH secretion in VLDL. Timefurone and khellin at therapeutic (10 μg/ml) concentration did not alter ^{125}I-LDL uptake and degradation, whereas both agents reduced the VLDL secretion and total release of apo-B; secretion of apo-E in VLDL remaining unchanged. A marked inhibition (by 30–50%) of CH synthesis was observed for both drugs. These results suggest that khellin and timefurone can exert their hypolipidemic action by a simultaneous decrease of hepatic CH synthesis and secretion of apo-B containing VLDL particles.

Introduction

Khellin was originally isolated from the Mediterranean plant, *Ammi visnaga* (Fantl and Salem, 1930), and belongs to the group of compounds called furochromones. The hypolipidemic and antiatherogenic potency of khellin was demonstrated recently for different species of animals (Day et al., 1985; Stevens et al., 1985a, b). It reduced the plasma total CH content by lowering the level of LDL (Stevens et al., 1985a, b). This drug usually decreases the CH level of VLDL in male SEA Japanese quail and rats (Stevens et al., 1985b) and increases it in male cynomolgus monkeys fed a CH-rich diet (Stevens et al., 1985a). Khellin was previously tried as a vasodilator, but some adverse effects prevented its general clinical application (Anrep et al., 1949; Conn et al., 1952). Timefurone is a khellin analog (Fig. 1) which looks promising for further clinical experimentation, because it retains the beneficial CH-lowering and antiatherosclerotic potentials and has no toxic side effects (Stevens et al., 1985a, b).

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Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; ME medium, minimal essential medium; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; Apo, apolipoprotein; CH, cholesterol; ECH, cholesterol esters; SDS, sodium dodecyl sulfate.

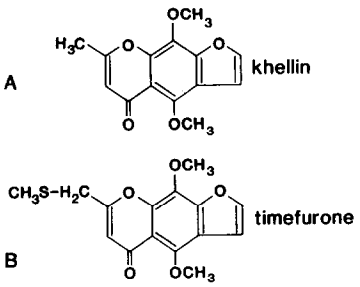

Fig. 1. Structure of khellin (A) and timefurone (B) molecules.

The underlying mechanism of the hypolipidemic effect of timefurone and khellin remains unknown. The present studies were undertaken to examine the influence of these compounds on the hepatic CH and lipoprotein metabolism in primary cultures of rabbit and human hepatocytes described in detail elsewhere (Kosykh et al., 1987).

Materials and Methods

Drugs and chemicals

All chemicals used were reagent grade. Minimal essential medium with Earle's salts (ME medium), heat-inactivated fetal calf serum (FCS), kanamycin, L-glutamine, culture dishes were purchased from Flow Laboratories, Inc. Collagenase type IY, 125–200 U/mg, was a product of Sigma Chemical Co., St. Louis, MO. Dispase II, 0.5 U/mg, was purchased from Boehringer (Mannheim, F.R.G.). L-[^{35}S]Methionine (spec. act. > 800 Ci/mmol) and [1-^{14}C]acetic acid, sodium salt (spec. act. 59 Ci/mol) were supplied by Amersham Corporation. Khellin and timefurone were kindly donated by the Upjohn Company.

Cultured hepatocytes

Rabbit hepatocytes were isolated from livers of chow-fed male rabbits (1.5–2 kg) by the method described elsewhere (Reese and Byard, 1981). Human hepatocytes were isolated according to Strom et al. (1982) from livers which were taken from renal donors aged 30 to 50 years. Cells were plated in culture dishes at 2×10^5 cells/cm^2 in ME medium containing FCS (10%, v/v), kanamycin (100 μg/ml), non-essential amino acids (1 mM) and were maintained at 38°C in 95% air–5% CO_2 atmosphere. Hepatocytes were cultured for 48 h under these conditions prior to use in metabolic studies. Percentage viability of the hepatocyte monolayer was determined by the Trypan blue test (Kosykh et al., 1987). The viability was greater than 85% over 18 h of cell maintenance with and without drugs.

Preparation and radioiodination of lipoproteins

Human and rabbit LDL (d 1.019–1.063 g/ml) were isolated from plasma by preparative ultracentrifugation (Havel et al., 1955) using a AH-650 rotor. The lipoproteins were washed twice by the reflotation on a AH-650 rotor and dialysed exhaustively against phosphate-buffered saline (pH 7.4), 0.01% EDTA. The purity of LDL was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis; they contain almost no other protein than apo-B. LDL were labeled using the iodine monochloride technique as described previously (Bilheimer et al., 1972) after which the lipoproteins were dialyzed (spec. act. 100–300 cpm/ng LDL protein).

Measurement of lipoprotein catabolism

After being in culture for 48 h, cells were washed with ME medium and then incubated for 24 h with ME medium containing 10% lipoprotein deficient serum (LDS) in the presence of 0.1, 1 and 10 μg/ml drugs. Thereafter ^{125}I-LDL were added into fresh medium at the 100 μg/ml concentration. Cells were incubated at 38°C for 18 h and then the medium was harvested for determination of ^{125}I-LDL degradation, which was assessed by quantitating the acid-soluble radioactivity in the medium (Goldstein et al., 1976). The acid-soluble radioactivity in the cell culture medium was corrected for acid-soluble ^{125}I generated in parallel incubations in the absence of cells. To determine the cell ^{125}I-LDL uptake (binding and internalization), the hepatocytes were washed 5 times with ice-cold phosphate-buffered saline and dissolved in 1 M NaOH. Aliquots were taken for quantitation of radioactivity and for determination of protein content (Lowry et al., 1951).

Other metabolic studies

After 48 h in culture, the medium was changed to one with or without drugs. After 24 h of in-

cubation, the medium was changed to one labeled with [^{14}C]acetate (5 μCi/ml) or [^{35}S]methionine (40 μCi/ml) and after 3 h and 18 h of cultivation, cells and medium were harvested. VLDL isolation and analysis, determination of the secretion rates of VLDL [^{35}S]apoproteins and [^{14}C]lipids, lipid analysis and incorporation of [^{14}C]acetate into cellular CH were carried out as in Kosykh et al. (1987), and the apo-B level assay as described by Preobrazhensky et al. (1985).

Data analysis

Results were analysed with Student's *t*-test and expressed as mean $\pm$ S.E. Values of $P < 0.05$ (two-tailed) were considered significant.

Results

As shown in Table 1, the 24-h preincubation of cells in the presence of 10 μg/ml drugs did not alter the uptake and degradation of ^{125}I-LDL. There was also no difference in ^{125}I-LDL uptake and degradation by human and rabbit hepatocytes incubated with 0.1 and 1 μg/ml drugs (data not shown).

TABLE 1

Effect of khellin and timefurone on the uptake and degradation of ^{125}I-LDL by human and rabbit hepatocytes

Data represent mean $\pm$ S.E. for a triplicate incubation.

	^{125}I-LDL uptake	^{125}I-LDL degradation
	ng/mg cell protein/18 h	
Rabbit cells		
Exp. 1		
control	501 $\pm$ 39	260 $\pm$ 30
khellin	454 $\pm$ 41	282 $\pm$ 32
timefurone	468 $\pm$ 63	278 $\pm$ 21
Exp. 2		
control	808 $\pm$ 48	281 $\pm$ 36
khellin	835 $\pm$ 62	243 $\pm$ 32
timefurone	913 $\pm$ 81	245 $\pm$ 25
Human cells		
control	616 $\pm$ 32	318 $\pm$ 20
khellin	689 $\pm$ 18	300 $\pm$ 22
timefurone	668 $\pm$ 32	320 $\pm$ 11

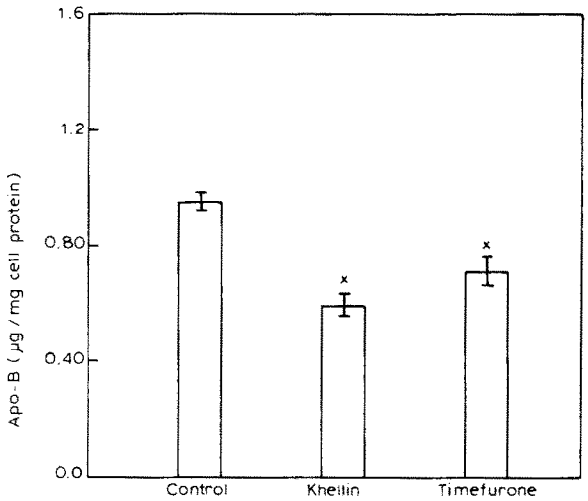

Fig. 2. Effect of timefurone and khellin on the rate of apo-B secretion by human hepatocytes. After 48 h in culture the ME medium (10% FCS) was changed to one containing 10% LDS with and without 10 μg/ml drugs. After an 18 h cultivation the medium apo-B level was determined by ELISA. The results represent the means $\pm$ SD of four replicates. Statistical significance from control * $P < 0.05$.

The data presented in Table 2 demonstrate that secretion of CH and ECH in VLDL by human and rabbit hepatocytes during an 18 h incubation with khellin and timefurone was reduced by 40–50% at a drug concentration of 10 μg/ml. To test whether the observed inhibition of VLDL lipid release could be caused by drug effects on the CH biosynthesis, we studied the incorporation of [^{14}C]acetate into cellular CH. From Table 2 it can be seen that tracer incorporation into CH was diminished by 30–50% after a 24 h exposure to 1 and 10 μg/ml of drugs.

The VLDL fractions secreted by cells in the absence or presence of drugs were treated by SDS-polyacrylamide gel electrophoresis and the [^{35}S]methionine incorporation into apo-B and apo-E were quantified. The results of this analysis are given in Table 3. At 10 μg/ml, khellin and timefurone inhibited the incorporation of [^{35}S]methionine into the VLDL apo-B. Fig. 2 shows that both khellin and timefurone lowered the secretion of the total medium apo-B as measured by ELISA. However, in the case of the VLDL apo-E there was no effect. Besides, there

TABLE 2

Effect of khellin and timefurone on VLDL lipid secretion, cellular lipid content and incorporation of [^{14}C]acetate into cellular CH of rabbit and human hepatocytes

The results represent the means ± S.E. of three replicates for each treatment and are expressed as cpm/mg cell protein and μg/mg cell protein. Statistical significance from control * $P < 0.05$.

Additions	Cell			Medium VLDL	
	CH	CH	ECH	CH	ECH
	(10^{-3} cpm/mg/3 h)	(μg/mg/18 h)		(μg/ml/18 h)	
Rabbit cells					
Exp. 1					
control	53.0 ± 0.8	30.2 ± 2.1	9.2 ± 0.8	2.2 ± 0.2	4.8 ± 0.3
1 μg/ml khellin	35.8 ± 1.6 *	26.0 ± 2.2	8.1 ± 0.7		
10 μg/ml khellin	33.8 ± 1.7 *	25.6 ± 1.8	8.2 ± 0.5	1.3 ± 0.1 *	3.2 ± 0.3 *
1 μg/ml timefurone	30.2 ± 2.1 *	33.5 ± 2.4	9.7 ± 1.0		
10 μg/ml timefurone	29.4 ± 1.4 *	28.9 ± 1.6	9.3 ± 0.9	1.4 ± 0.1 *	3.4 ± 0.3 *
Exp. 2					
control	58.1 ± 3.1	14.8 ± 1.5	6.2 ± 0.3	1.4 ± 0.1	6.2 ± 0.7
1 μg/ml khellin	42.1 ± 1.8 *				
10 μg/ml khellin	21.1 ± 1.7 *	13.3 ± 1.0	5.1 ± 0.4	0.6 ± 0.1 *	2.9 ± 0.2 *
1 μg/ml timefurone	52.2 ± 5.0				
10 μg/ml timefurone	33.6 ± 2.8 *	14.7 ± 1.1	5.7 ± 0.3	0.7 ± 0.1 *	3.7 ± 0.3 *
Human cells					
control	38.1 ± 0.6			3.7 ± 0.3	0.9 ± 0.1
10 μg/ml khellin	20.2 ± 2.1 *			2.4 ± 0.2 *	0.5 ± 0.1 *
10 μg/ml timefurone	22.2 ± 3.2 *			2.6 ± 0.2 *	0.6 ± 0.1 *

TABLE 3

Incorporation of [^{35}S]methionine into apo-B and apo-E of VLDL secreted by rabbit and human hepatocytes

The results represent the means ± S.E. of three replicates. Statistical significance from control * $P < 0.05$.

Additions	Apo-B	Apo-E
	10^{-2} cpm/mg cell protein	
Rabbit cells		
Exp. 1		
control	47.9 ± 3.2	15.5 ± 1.4
khellin (10 μg/ml)	33.3 ± 3.1 *	13.5 ± 1.6
timefurone (10 μg/ml)	31.5 ± 2.8 *	14.6 ± 0.8
Exp. 2		
control	49.5 ± 4.1	41.4 ± 3.8
khellin (10 μg/ml)	29.5 ± 3.4 *	40.5 ± 3.6
timefurone (10 μg/ml)	29.4 ± 2.5 *	36.4 ± 3.2
Exp. 3		
control	31.7 ± 2.8	49.8 ± 4.0
khellin (10 μg/ml)	24.8 ± 2.2 *	46.4 ± 4.7
Human cells		
control	31.4 ± 2.5	17.8 ± 0.1
khellin	18.4 ± 0.2 *	18.3 ± 1.4
timefurone	15.8 ± 0.9 *	22.0 ± 1.2

was no significant difference in the total protein secretion by human and rabbit hepatocytes incubated with drugs as evaluated by the [^{35}S]methionine incorporation into trichloroacetic acid-precipitable materials (data not shown).

Discussion

Several observations suggest that timefurone may be one of the drugs for hypercholesterolemia treatment (Stevens et al., 1985a, b). However, the mechanism whereby timefurone can reduce the CH level in the LDL fraction has not been elucidated. Two major possibilities should be considered in this connection, namely, a stimulation of the hepatic LDL uptake or changes in the VLDL production. Our data do not support the proposition that in animals timefurone is able to lower the apo-B associated CH by enhancing the uptake and degradation of LDL by hepatocytes (Table 1). However, here only short-term experiments have

been performed on primary cultures. Therefore, a different mode of drug action, in prolonged experiments, imitating more closely the situation in vivo cannot be excluded.

Another route whereby timefurone and khellin might exert their hypolipidemic action is to inhibit the hepatic CH synthesis. To our knowledge no indication of the influence of these substances on the CH biosynthesis has been published so far. Presently we report that both these drugs can inhibit the cellular synthesis of CH and CH secretion in VLDL at therapeutic concentration (10 μg/ml). Based on these data one can offer an explanation for the plasma CH lowering effect of orally administered drugs in animals. In addition, the effect of drugs in human hepatocytes testifies that the drugs can inhibit the CH synthesis in human liver as well.

We have also examined whether the drugs influence the CH secretion in VLDL by an alternative mechanism. Since the major protein of VLDL and LDL is apo-B and the availability of apo-B may limit VLDL secretion (Davis and Boogaerts, 1982), we have measured the release of the ^{35}S-labeled apo-B in the VLDL and found that both substances inhibited this process. Besides, khellin and timefurone also reduced the total apo-B secreted by human hepatocytes. Hence, the lower CH level in the VLDL fraction could be attributed simply to the decrease in the number of VLDL particles. Here it was also found that apo-E secretion in VLDL was not affected by drugs. This disparity between the secretion patterns of the newly synthesized apo-B and apo-E is not yet understood. The apolipoprotein heterogeneity of VLDL secreted by cultured hepatocytes may be mentioned as one of the possible causes. Indeed, recently it was found (Dashti et al., 1987) that human hepatoma cells Hep G-2 secreted apo-B containing lipoprotein particles of two classes: apo-B and apo-B,E-containing particles. Therefore, the drugs studied may influence the secretion of the apo-B VLDL particles and apo-B,E VLDL particles differently. The investigation of such an assumption is currently under way.

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