Inhibition of apolipoprotein B net synthesis and secretion from cultured rat hepatocytes by the calcium-channel blocker diltiazem

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1. The effect of the Ca^{2+} -channel blocker diltiazem on hepatic apolipoprotein B (apo B) synthesis and secretion was studied in 12–18 h cultures of collagenase-dispersed rat hepatocytes. 2. The presence of diltiazem in the medium decreased apo B secretion by hepatocytes in a concentration-dependent manner. At 25 μM , diltiazem inhibited apo B secretion by approx. 36 %, but there was no evidence of intracellular accumulation of apo B. 3. The inhibition of apo B secretion by hepatocytes was significantly correlated with cell-associated diltiazem ($r = 0.72$, $P < 0.01$). 4. The rate of apo B secretion remained linear over 16 h even in the presence of 50 μM -diltiazem. 5. At diltiazem concentrations in the medium which were inhibitory for apo B secretion, [^{14}C]acetate incorporation into cellular lipids and [^{35}S]methionine incorporation into protein were enhanced. 6. Diltiazem inhibited the secretion of the apo B variants with a preferential inhibition of the higher-molecular-mass form of apo B (apo B_H) over the lower-molecular-mass form (apo B_L) at diltiazem concentrations in the medium greater than 25 μM . 7. Together, these results suggest that Ca^{2+} may play an important role in the synthesis and secretion of apo B-containing lipoproteins.

INTRODUCTION

Ca^{2+} antagonists including verapamil have been shown to inhibit VLDL triacylglyceride secretion by primary cultures of rat hepatocytes in 2 h cultures (Nossen *et al.*, 1987). The inhibition of triacylglycerol secretion by verapamil occurred within 30 min of incubation, was reversible and could not be explained by the decreased synthesis of triacylglycerol or apolipoproteins. These results, in the context of other reports (Rustan *et al.*, 1987), imply a role for Ca^{2+} in the secretion of VLDL triacylglycerol. In the present studies we report the long-term effects of the Ca^{2+} -channel blocker diltiazem on the net synthesis and secretion of apo B in 12–18 h cultures of rat hepatocytes. Apo B was examined because of its necessary role in the assembly and secretion of triacylglycerol-rich lipoproteins (Gotto *et al.*, 1971). Our results demonstrate that diltiazem is quickly taken up by hepatocytes while inhibiting apo B secretion in a concentration-dependent manner. Diltiazem stimulates protein synthesis from methionine and triacylglycerol synthesis from acetate by hepatocytes, but no significant accumulation of apo B occurs within cells. These results suggest that the long-term effect of diltiazem is mediated at the level of assembly of apo B with triacylglycerol. Protein labelling studies suggest a direct diltiazem effect on apo B synthesis, indicating a role for cellular Ca^{2+} in the regulation of the hepatic apo B synthesis/secretion pathway.

EXPERIMENTAL

Materials

Diltiazem was from Marion Laboratories (Kansas City, MO, U.S.A.), and [^3H]diltiazem (78.6 Ci/mmol)

was from New England Nuclear (Boston, MA, U.S.A.). [^{14}C]Acetate (1–3 mCi/mmol) and L-[^{35}S]methionine (1115–1483 Ci/mmol) were from Amersham International (Arlington Heights, IL, U.S.A.). Waymouth's MB 752/1 medium, gentamicin and penicillin were from Gibco Laboratories (Grand Island, NY, U.S.A.). RPMI 1640 medium, Triton X-100, L-methionine and benzamidine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase (Type II) was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Culture dishes were from Costar (Cambridge, MA, U.S.A.). Fixed *Staphylococcus aureus* cells were supplied as Immunoprecipitin in the form of a 10 % (w/v) suspension, from Bethesda Research Laboratories (Bethesda, MD, U.S.A.) All other chemicals were of the highest purity available from commercial sources.

Preparation and incubation of hepatocytes

Hepatocytes were prepared from male Sprague-Dawley rats (200–250 g) by collagenase perfusion and plated in serum-free medium as described previously (Sparks *et al.*, 1986a).

Experiments with hepatocytes

Hepatocytes were placed on 60 mm-diam. sterile Petri dishes coated with rat tail collagen (70 μg /dish), and the dishes were incubated for 3 h at 37 °C in an atmosphere of air/ CO_2 (19:1). Afterwards, medium and non-adherent cells were discarded and adherent cells washed with 3 $\times$ 2 ml of wash buffer, containing 10 mM-sodium phosphate, pH 7.4, 0.14 M-NaCl and 0.1 % (w/v) BSA. Fresh Waymouth's medium (2 ml per 60 mm dish) containing 0.2 % BSA was added to washed monolayers, and cells were incubated for various time periods with

Abbreviations used: VLDL, very-low-density lipoprotein; apo B, apolipoprotein B; apo B_H, apolipoprotein B of higher molecular mass; apo B_L, apolipoprotein B of lower molecular mass; BSA, bovine serum albumin.

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medium containing diltiazem. Control plates received the same volume of buffer without added drug.

Determination of cell and medium apo B

After incubation, media were removed and apo B was quantified by solid-phase radioimmunoassay using a purified mouse ^{125}I -labelled anti-(rat apo B) monoclonal antibody as described in detail elsewhere (Sparks *et al.*, 1986b). For cellular apo B, hepatocytes were homogenized in a buffer containing 0.5% (v/v) Triton X-100 (Patsch *et al.*, 1983), and samples were used in the monoclonal-antibody immunoassay with VLDL apo B prepared in homogenizing buffer as standard (Sparks *et al.*, 1988). The protein content of plates was determined with BSA as standard (Markwell *et al.*, 1978).

Determination of cell-associated drug

Cell-associated [^3H]diltiazem was measured after washing the cells with unlabelled medium three times. Radioassay of cell extracts was corrected for quench, and specific radioactivity of the drug was used to calculate the amount of drug taken up by hepatocytes. Control experiments demonstrated that less than 0.1% (w/v) of the labelled drug added was associated with the plastic tissue-culture dishes.

Determination of cellular lipid and protein synthesis

For cellular lipid studies, medium contained 1 μCi of [^{14}C]acetate (2 Ci/mol, 0.5 mM-acetate)/ml, and cells were incubated for 2 h. After incubation, cells were washed three times in unlabelled medium and scraped into extraction tubes. Cellular lipids (approx. 1 mg of cell protein) were extracted in 2.0 ml of chloroform/methanol (1:1, v/v). Extracts were clarified by centrifugation and an additional 1 vol. of chloroform was added. The extract was partitioned by addition of 0.4 ml of 0.1 M-sodium acetate. The chloroform layer was removed, evaporated and radioassayed. For total protein-synthesis studies, media contained 7.5 μCi of [^{35}S]methionine (75 Ci/mol, 0.1 mM)/ml. After 6 h of incubation with label, monolayers were washed three times with unlabelled medium and the amount of [^{35}S]methionine incorporated into cellular protein was determined by precipitation of cell extracts with 10% (w/v) trichloroacetic acid as described previously (Sparks *et al.*, 1986a).

Immunoprecipitation of ^{35}S -labelled apo B from media

Hepatocytes were incubated in RPMI medium containing 0.2% bovine serum albumin and [^{35}S]methionine (sp. radioactivity 33 Ci/mmol) (100 μCi /dish, 1.1 μM -L-methionine) for 12 h. Afterwards, medium and cells were separated and the medium was clarified by centrifugation. Apo B in the medium was quantitatively immunoprecipitated with rabbit anti-(rat apo B) sera. Medium (0.5 ml) was diluted with an equal volume of 0.05 M-Tris/HCl/0.15 M-NaCl buffer, pH 7.4, containing 1% Triton X-100, 0.5% (w/v) SDS, 5 mM-EDTA, 1 mM- α -toluenesulphonyl fluoride and 2 mM-benzamidine. After mixing, 0.5 ml of antiserum diluted in 1% BSA/phosphate-buffered saline was added. The solution was thoroughly mixed and incubated overnight at 4 °C. Rabbit antisera were prepared by immunizing rabbits with purified apo B_H and apo B_L isolated from $d < 1.063$ rat lipoproteins by SDS-column chromatography (Sparks & Marsh, 1981a). After overnight incubation, 25 μl of a 10% (w/v) suspension of fixed *Staphylococcus aureus* cells was

added, and incubation was continued for an additional 30 min at room temperature. The immunoprecipitate was collected by centrifugation and washed three times in 0.01 M-Tris/HCl/0.15 M-NaCl buffer, pH 7.4, containing 0.15% SDS, 0.1% Triton X-100 and 2 mM-EDTA. Afterwards, labelled apo B was eluted by adding 100 μl of 0.06 M-Tris/HCl buffer, pH 6.8, containing 2% SDS, 5% (v/v) 2-mercaptoethanol, 5 mM-dithiothreitol and 10% (v/v) glycerol and heating the solution twice to 95 °C. Bacterial cells were removed by centrifugation, and labelled apo B was applied to an Acrylaide/3.5–24%-acrylamide gradient gel. Apo B isoforms were then separated by SDS/polyacrylamide-gel electrophoresis (Sparks *et al.*, 1986a). After enhancement of the gels, labelled apo B isoforms were observed by macro-fluorography (Sparks *et al.*, 1988). Films were pre-flashed to linearize film exposures, and intensities of bands were monitored to assure a linear response of radioactivity to absorbance readings. Films were processed by an automatic processor, and density of each band was measured with an automated integrating gel scanner.

RESULTS

Cellular uptake of diltiazem

Rat hepatocyte uptake of diltiazem after incubation with the drug for 6 h was dependent on diltiazem concentration in the medium. The uptake was saturable at drug concentrations in media above 45 μM (Fig. 1), and the cell-associated diltiazem (nmol/mg of cell protein) at saturating conditions represented approx. 5% of the total drug added. At a non-saturating diltiazem concentration of 25 μM , hepatocellular uptake of diltiazem consisted of a very rapid initial phase (< 1 h), followed by a slower phase (0.61 ± 0.13 nmol/h per mg of cell protein; mean $\pm$ S.E.M.) that remained linear for at least 8 h. The amount of cell-associated diltiazem reached a plateau by 16 h.

Fig. 1. Effect of diltiazem concentration in the medium on amount of cell-associated drug

Primary cultures of rat hepatocytes were incubated for 16 h in Waymouth's medium. After overnight incubation, the cells were washed three times and re-incubated with media containing various concentrations of [^3H]diltiazem (0–68 μM) for 6 h. Afterwards, the cells were washed and cell-associated radioactivity was determined. Cell-associated diltiazem (nmol/mg of cell protein) is plotted against initial diltiazem concentration in the medium. Results are expressed as means $\pm$ S.E.M. for three separate liver preparations.

Table 1. Effect of diltiazem concentration in the medium on apo B secretion by primary cultures of rat hepatocytes

Hepatocytes were prepared from normal rats and incubated overnight in Waymouth's medium containing 0.2% BSA and 0, 10 μM -, 25 μM -, 50 μM - and 75 μM -diltiazem in triplicate plates of cells. After 6–8 h and 12–14 h of incubation, media were removed and apo B was assayed in triplicate on each medium sample. The apo B concentrations at various medium concentrations of diltiazem are expressed as a percentage of medium apo B concentration in hepatocytes incubated in the absence of diltiazem. Results are expressed as the mean percentages $\pm$ S.E.M., and the numbers of different hepatocyte preparations from separate livers are shown in parentheses. Values marked * and ** are significantly different from the no-added-diltiazem control at $P < 0.001$ and $P < 0.02$ respectively. ND, not determined.

Diltiazem (μM)	Duration of culture...	Inhibition (%)	
		6–8 h	12–14 h
0		100	100
10		88 $\pm$ 12.0 (4)	84 $\pm$ 8.3 (3)
25		63 $\pm$ 5.6 (11)*	67 $\pm$ 6.0 (6)*
50		48 $\pm$ 14.0 (3)**	51 $\pm$ 5.0 (4)*
75		48 $\pm$ 3.7 (7)*	ND

Inhibition of apo B secretion by diltiazem

The presence of diltiazem in the medium significantly decreased the secretion of apo B (Table 1). At 25 μM - and 50 μM -diltiazem, the rate of apo B secretion remained linear over 16 h of incubation. The percentage inhibition of apo B secretion was dependent on the diltiazem concentration in the medium (0–68 μM) and was also significantly correlated with cell-associated diltiazem ($r = 0.71$, $P < 0.01$). Linear-regression analysis yielded the line $y = 91.7 - 6x$, where y is the percentage of apo B secreted relative to control hepatocytes and x is cell-associated diltiazem in nmol/mg of cell protein, based on

results from four livers and 55 plates of hepatocytes. Despite an inhibition of apo B secretion by as much as 36% by 25 μM -diltiazem, there was no evidence of intracellular accumulation of apo B. Cellular apo B in cells incubated in 25 μM -diltiazem, as a percentage of that in hepatocytes incubated in the absence of diltiazem, was $94 \pm 10.5\%$ (S.E.M.) in three liver preparations where apo B was assayed in triplicate sets of plates at each condition.

Effect of diltiazem on cellular lipid and protein synthesis

Diltiazem in the medium stimulated [^{14}C]acetate incorporation into cellular lipids. At diltiazem concentrations of 50 μM in the medium, there was a 2-fold increase in cellular lipid synthesis (Table 2). Diltiazem at concentrations of 10 and 25 μM in the media stimulated total protein synthesis, as assessed by [^{35}S]methionine incorporation into cellular proteins (Table 3). Diltiazem-stimulated hepatocytes, however, were able to maintain the ratio of [^{35}S]methionine-labelled cellular to secreted media proteins.

Effect of diltiazem on synthesized and secreted apo B isoforms

The inhibitory effect of diltiazem on total apo B is also evident on the two apo B isoforms, apo B_H and apo B_L. There was a concentration-dependent inhibition by diltiazem on secretion of [^{35}S]methionine-labelled apo B_H and apo B_L (Fig. 2 and Table 4). At initial diltiazem concentrations greater than 25 μM , diltiazem appeared to have a differential effect on the inhibition of apo B_H and apo B_L secretion (Table 4), that of apo B_H being more prominently inhibited at the higher diltiazem concentrations used.

DISCUSSION

The current study was undertaken to investigate the long-term effects of the Ca^{2+} -channel blocker diltiazem on apo B secretion in primary cultures of rat hepatocytes. In rats, there are two variants of apo B secreted in VLDL by rat liver (Krishnaiah *et al.*, 1980; Sparks *et al.*, 1981; Elovson *et al.*, 1981; Wu & Windmueller, 1981),

Table 2. Effect of diltiazem concentration in the medium on the incorporation of [^{14}C]acetate into cellular lipids by primary rat hepatocyte cultures

Hepatocytes were prepared from normal rats and incubated overnight in Waymouth's medium containing 0.2% BSA. After 12 h of incubation, cells were washed three times in medium and re-incubated in Waymouth's medium containing 1 μCi of [^{14}C]acetate (2 Ci/mol) for 2 h. At the end of the incubation period, cells were washed three times in phosphate-buffered saline and cellular lipid radioactivity was determined as described in the Experimental section. Values are expressed as the lipid synthesis by hepatocytes incubated with various diltiazem concentrations as a percentage of that observed with hepatocytes incubated in the absence of diltiazem. Lipid analyses were performed in triplicate, and the numbers of different hepatocyte preparations from separate livers are shown in parentheses. Values marked * are significantly different ($P < 0.04$) from those obtained for cells incubated in the absence of diltiazem. ND, not determined.

Diltiazem (μM)	Lipid synthesis by hepatocytes (% of control hepatocytes)				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Mean $\pm$ S.E.M.
0	100	100	100	100	—
10	118	138	170	112	135 $\pm$ 13 (4)*
25	128	158	ND	130	139 $\pm$ 10 (3)*
50	171	227	228	170	199 $\pm$ 17 (4)*

Table 3. Effect of diltiazem concentration in the medium on total, cell and medium protein synthesis from [³⁵S]methionine by primary cultures of rat hepatocytes

Hepatocytes were prepared from normal rats and incubated overnight in Waymouth's medium containing 0.2% BSA and 0, 10 μ M-, 25 μ M-, 50 μ M- and 100 μ M-diltiazem in triplicate plates of cells. Protein synthesis was assayed by the incorporation of [³⁵S]methionine (15 μ Ci/dish), which was included in the culture medium as described in the Experimental section. After 12–14 h of incubation the experiment was terminated, and label incorporated into cell, medium and total protein was determined by trichloroacetic acid precipitation as described in the Experimental section. Values at the various diltiazem concentrations are expressed as percentages of total protein synthesized in the absence of diltiazem. Results are expressed as the mean percentage $\pm$ S.E.M., for three separate livers. Values marked * are significantly different ($P < 0.05$) from those obtained for hepatocytes incubated in the absence of diltiazem.

Diltiazem (μ M)	Protein synthesized from [³⁵ S]methionine (%)		
	Total	Cell	Medium
0	100	70 $\pm$ 5.0	30 $\pm$ 5.0
10	125 $\pm$ 7.0*	85 $\pm$ 5.9	41 $\pm$ 6.4
25	124 $\pm$ 8.2*	78 $\pm$ 5.1	46 $\pm$ 9.6
50	116 $\pm$ 9.3	75 $\pm$ 3.8	42 $\pm$ 13.0
100	120 $\pm$ 14.6	70 $\pm$ 4.0	51 $\pm$ 12.2

which have been designated as the higher-molecular-mass form apo B_H and the lower-molecular-mass form apo B_L (Sparks & Marsh, 1981a,b). Apo B secretion was specifically examined, as it is an essential component of VLDL, being required for VLDL assembly with triacylglycerol and subsequent lipoprotein secretion (Gotto *et al.*, 1971). By studying the effect of diltiazem on apo B in cells and medium, the distinction between inhibition of secretion of pre-assembled VLDL and inhibition of newly synthesized VLDL apo B can be made. Also, specific effects of diltiazem on apo B_H versus apo B_L can be documented. This becomes important, as particle composition may affect the rate at which VLDL are metabolized by peripheral cells (Sparks & Marsh, 1981b; Sparks *et al.*, 1981, 1983).

Previous reports using primary cultures of rat hepatocytes have indicated that VLDL triacylglycerol secretion was inhibited by Ca²⁺ antagonists, including verapamil (Nossen *et al.*, 1987; Rustan *et al.*, 1987). The inhibition of triacylglycerol secretion by verapamil was evident within 30 min, was reversible and was dose-dependent, with a maximum of 90% inhibition at a concentration of 200 μ M. The mechanism of action of verapamil (Rustan *et al.*, 1987) was suggested to be the inhibition of secretion of already assembled VLDL in Golgi complexes. As similar results were obtained with chloroquine, and both are weak bases, it was suggested that these compounds accumulate in acidic vesicles, alter the pH and subsequently affect VLDL secretion. This hypothesis implies that an acidic environment of trans-Golgi vesicles and cisternae must be important in the secretion of assembled VLDL triacylglycerol (Nossen *et al.*, 1984; Rustan *et al.*, 1985, 1987). Monensin, an

Fig. 2. Fluorograph of [³⁵S]methionine-labelled apo B isoforms secreted into 12 h culture medium at various initial concentrations of diltiazem

Primary cultures of rat hepatocytes were incubated in medium containing 100 μ Ci of [³⁵S]methionine per plate and diltiazem (0–200 μ M) for 12 h. After the incubation period, media were collected and apo B was specifically immunoprecipitated with a rabbit anti-(rat apo B) polyclonal antiserum. Immunoprecipitated isoforms of apo B were separated electrophoretically by SDS/polyacrylamide-gel electrophoresis, and gels were enhanced and fluorographs prepared. Numbers in the Figure refer to the following initial diltiazem concentrations: 1, 0; 2, 10 μ M; 3, 25 μ M; 4, 50 μ M; 5, 100 μ M; 6, 200 μ M. Positions of molecular-mass markers (kDa) are shown to the right; O, origin; S, start of separating gel.

Table 4. Effect of diltiazem concentration in the medium on the secretion of labelled apo B_H and apo B_L by primary cultures of rat hepatocytes

Hepatocytes were prepared from normal rats and incubated overnight in Waymouth's medium containing 0.2% BSA, [³⁵S]methionine (100 μ Ci/dish) and 0, 10 μ M-, 25 μ M-, 50 μ M- and 100 μ M-diltiazem. Medium apo B synthesized and secreted was determined by immunoprecipitation and separation of apo B forms by SDS/polyacrylamide gel electrophoresis and fluorography of enhanced gels as described in the Experimental section. Values at the various diltiazem concentrations are expressed as the percentage of apo B synthesized and secreted in medium in the absence of diltiazem. Results are expressed as the mean percentage $\pm$ S.E.M., for three separate livers. Values marked * are significantly different ($P < 0.05$) from those obtained for hepatocytes incubated in the absence of diltiazem.

Diltiazem (μ M)	Secretion of ³⁵ S-labelled apo B (%)		
	Apo B _H	Apo B _L	Total apo B
0	100	100	100
10	79 $\pm$ 11.7	74 $\pm$ 2.8*	77 $\pm$ 3.4*
25	51 $\pm$ 6.9*	57 $\pm$ 5.6*	55 $\pm$ 5.9*
50	23 $\pm$ 3.0*	36 $\pm$ 8.3*	29 $\pm$ 7.9*
100	10 $\pm$ 1.6*	23 $\pm$ 5.0*	18 $\pm$ 3.0*

ionophore that disrupts proton gradients and alters the normal function of the Golgi apparatus, likewise inhibits VLDL triacylglycerol secretion (Rustan *et al.*, 1987).

The current study demonstrates that the Ca^{2+} -channel blocker diltiazem becomes rapidly associated with rat hepatocytes in a dose-dependent fashion and that cell-associated diltiazem correlates with the inhibition of apo B secretion. The effect of diltiazem on apo B secretion was evident at diltiazem concentrations in the medium as low as $10\text{ }\mu\text{M}$. At $50\text{ }\mu\text{M}$ -diltiazem apo B secretion was inhibited by approx. 50% in 12 h cultures. The inhibition of apo B secretion was not due to a general inhibitory effect of diltiazem on protein synthesis, because in control studies the amount of radiolabelled methionine incorporated into newly synthesized cell protein was actually slightly stimulated by diltiazem. Concurrent with the inhibition of apo B secretion was the stimulation of acetate incorporation into cellular lipid. Diltiazem in the medium ($25\text{ }\mu\text{M}$) did not result in the accumulation of intracellular apo B in 12–14 h cultures, indicating that the diltiazem effect was not a simple blockade of secretory vesicles containing assembled VLDL, as suggested by the previous short-term studies with verapamil.

Diltiazem inhibited the secretion of both variants of rat apo B. At drug concentrations greater than $25\text{ }\mu\text{M}$, there was greater inhibition of apo B_H secretion than of apo B_L . These results suggest a selective action of diltiazem on the apo B variant which ultimately forms plasma low-density lipoprotein (Sparks *et al.*, 1983). The inhibitory effect of diltiazem on apo B secretion by rat hepatocytes is apparent only when cells have been exposed to diltiazem concentrations greater than $10\text{ }\mu\text{M}$. This concentration is 25 times the steady-state serum concentration ($0.4\text{ }\mu\text{M}$) observed in patients treated with diltiazem (Hung *et al.*, 1988). However, diltiazem concentration within the liver could be much higher than that in blood, because the liver is the major organ of drug metabolism and the hepatic extraction ratio for diltiazem is relatively high (0.54; Kolle *et al.*, 1983).

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