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27-Hydroxycholesterol modulation of low density lipoprotein metabolism in cultured human hepatic and extrahepatic cells

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27-Hydroxycholesterol**, 25-hydroxycholesterol and cholesterol suppressed LDL uptake and degradation in human extrahepatic and hepatic cell lines in a concentration-dependent manner. Cholesterol was the least potent, and the inhibitory effect of oxysterols was more pronounced in skin fibroblasts and in endothelial cell line EAhy 926 than in hepatoma HepG2 cells. Shorter incubations were required for oxysterols to achieve 50% inhibition of LDL uptake and degradation in all cultured cells. The inhibition of LDL catabolism in extrahepatic cells by 27-hydroxycholesterol occurred at concentrations close to those observed in human plasma (0.2–0.6 μM). The results support a possible role of 27-hydroxycholesterol, a physiological oxysterol, in the regulation of cellular cholesterol homeostasis in non-hepatic tissues.

Oxysterol; LDL receptor; Human hepatoma cell line Hep G2; Human endothelial cell; Human fibroblast

1. INTRODUCTION

Oxygenated derivative(s) of cholesterol are believed to play a role in the regulation of cellular cholesterol homeostasis [1–5]. Oxysterols are potent inhibitors of HMG-CoA reductase activity and hence of cholesterol biosynthesis [1,3,6,7]. The possibility exists that the actual modulator of cholesterol homeostasis is not cholesterol itself, but a more polar derivative either formed in the cell or produced by cellular oxidation of lipoprotein cholesterol. The hypothesis that exogenously added oxysterols are more potent inhibitors of HMG-CoA reductase because they more easily enter the cell and the regulatory pool of sterols than the less polar pure cholesterol cannot be ruled out. The demonstration, however, that the addition of purified cholesterol to cells does not influence HMG-CoA reductase activity [8,9], and the existence of a cytosolic binding protein for oxysterols but not for cholesterol [10–13], attest the role of an oxygenated sterol(s) as regulator(s) of cholesterol synthesis. Perhaps, one of the most convincing evidences supporting this proposal is that ketoconazole, a drug that inhibits cytochrome P_{450}-dependent hydroxylations, when added in vitro to non-hepatic cells, prevents the ability of LDL, but not that of hydroxysterols, to decrease the expression of LDL receptors [14–16]. These data suggest that the physiological regulator of cholesterol homeostasis is likely to be an oxygenated cholesterol species formed in a cytochrome P_{450}-catalyzed reaction. 25-Hydroxycholesterol and 27-hydroxycholesterol are among the most potent inhibitors of the HMG-CoA reductase [1,17–19] and, in addition, they decrease the receptor-mediated uptake and degradation of low density lipoprotein in cultured cells [17,20–22], thus interfering with both the endogenous and exogenous supply of cholesterol to the cells. 25-Hydroxycholesterol seems to be an autoxidation product of cholesterol and its presence in vivo is disputed [23–25]. Several groups have suggested 27-hydroxycholesterol to be a likely candidate [18,22,26,27]. 27-Hydroxycholesterol is synthesized from cholesterol by a cytochrome P_{450} containing mitochondrial enzyme (C27-steroid 27-hydroxylase) in most tissues [22,26,28], represents an intermediate in the synthesis of bile acids [22,29], is normally present in human plasma lipoprotein [22,30] and in healthy and atherosclerotic human aorta [31,32]. Recently, Saucier et al. [24], although not confirmed by others [33], reported the presence of increased levels of oxysterols (24-, 25- and 27-hydroxycholesterol) and repression of HMG-CoA reductase in cholesterol fed-mice according to the hypothesis that intracellular oxysterols regulate the level of the reductase.

Based on these premises, we compared the effect of 25-, 27-hydroxycholesterol and cholesterol on LDL re-

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**Recent publications have chosen to use 27-hydroxycholesterol rather than the conventional name 26-hydroxycholesterol to indicate that the mitochondrial enzyme stereospecifically hydroxylates only the methyl group in position C-27 [22].

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; LDL, low density lipprotein; 25-OH-C, 25-hydroxycholesterol; 27-OH-C, 27-hydroxycholesterol; BSA, bovine serum albumin; HSF, human skin fibroblasts; MEM, minimum essential medium; LPDS, lipoprotein deficient serum.

ceptor pathway in different human cell lines: skin fibroblasts and the permanent endothelial cell line EAhy 926 as models for extra-hepatic tissues, and the hepatoma cell line HepG2 as a model of liver parenchymal cells.

2. MATERIALS AND METHODS

2.1. *Materials*

$Na^{125}I$, [^{3}H]cholesterol, [^{14}C]oleic acid and [^{14}C]Na-acetate were from Amersham (Amersham, UK); all cell culture media and fetal calf serum were from Gibco (Grand Island, NY, USA); bovine serum albumin, cholesterol and 25-hydroxycholesterol were from Sigma (St. Louis, USA); 27-hydroxycholesterol was from Research Plus Inc. (Bayonne, NJ, USA); disposable sterile materials for cell cultures were from Corning (Corning, NY, USA).

2.2. *Cell culture*

Human skin fibroblasts were grown in monolayers from explants of skin biopsies obtained from normolipidemic clinically healthy volunteers, and maintained at 37°C in a humidified atmostphere of 5% CO_2 in minimum essential medium supplemented with 10% FCS, non-essential amino acid solution (1%, v/v), penicillin (100 U/ml), streptomycin (100 μg/ml), tricine buffer (20 mM, pH 7.4), $NaHCO_3$ (24 mM) [20]. The established human hepatoma cell line Hep G2 was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were grown in monolayers and cultured as described for HSF [20] with the addition to the medium of 0.11 g/l of sodium pyruvate [34]. The permanent human cell line, EAhy 926, was kindly provided by Dr. C.J.S. Edgell (North Carolina, USA) and was cultured as described for HSF with the addition to the medium of 1% HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) [35].

2.3. *Low-density lipoproteins and lipoprotein-deficient serum*

LDL (density 1.019–1.063 g/ml) were isolated from human serum by sequential preparative ultracentifugation [36], and were iodinated with ^{125}I by the McFarlane [37] monochloride procedure as modified for lipoproteins by Bilheimer et al. [38]. Specific activity of the labeled LDL was 115–220 cpm/ng protein and less than 2% of the radioactivity was trichloroacetic acid-soluble. Lipoproteins were immediately sterilized by filtration and stored at 4°C until use. LPDS was prepared by ultracentrifugation of human serum at density = 1.25 g/ml at 40,000 rpm for 72 h, and sterilized by filtration [39].

2.4. *Uptake and degradation of [^{125}I]LDL*

For all experiments, cells were seeded in 35 mm dishes (1.5×10^5 for HSF and EAhy 926 cells and 5×10^5 for Hep G2 cells) and used just before reaching confluency, usually 6 days after plating. Monolayers of cells were preincubated in a medium containing 5% lipoprotein-deficient serum to up-regulate the LDL receptors, for the indicated times, in the presence or absence of sterols. Sterols were dissolved in ethanol (1% final concentration); control cell dishes received the same volume of the solvent. The uptake (binding + internalization) of [^{125}I]LDL was evaluated as described by Goldstein et al. [40]. Briefly, after preincubation in a MEM containing 5% LPDS for the indicated times and concentrations of sterols, the cells were incubated with the same medium in the presence of [^{125}I]LDL (7.5 μg/ml) for 5 h. After this time, monolayers were chilled on ice, the media were collected for the measurement of LDL-degradation and the cells were washed five times with cold phosphate buffered saline containing BSA 2 mg/ml and twice again with PBS. Then the cells were digested in 0.1 M NaOH and aliquots were taken for LDL uptake determination. LDL degradation was measured as the accumulation of non-iodide trichloroacetic acid-soluble ^{125}I in the incubation medium in excess of that occurring in the absence of cells. Non specific uptake and degradation were determined by adding a 100-fold excess of unlabelled LDL [20,34]. Cellular protein content was evaluated according to Lowry et al. [41]. The amount of sterols required to inhibit 50% of [^{125}I]LDL degradation by cultured cells was calculated by linear regression analysis of the logarithm of the concentrations (μM) vs. probits and read from a probit transformation table [42].

3. RESULTS AND DISCUSSION

In preliminary experiments the tested cell lines, incubated in the absence of sterols, showed a specific [^{125}I]LDL uptake which was half-saturated at ligand concentrations of 12, 25 and 55 μg of lipoprotein protein/ml for HSF, EAhy 926, and Hep G2 cells, respectively. In the presence of sterols, at concentrations ranging between 0.4 and 100 μM, the uptake and degradation of [^{125}I]LDL decreased and this effect was more pronounced in HSF and EAhy 926 than in Hep G2 cells (Fig. 1). This action of the tested sterols was dose-dependent with IC_{50} values, evaluated as LDL degradation, of 0.02, 0.04 and 18 μM in endothelial cells and 3.1, 19 and higher than 100 μM in HepG2 cells for 25-OH-C, 27-OH-C and cholesterol, respectively.

In addition, as shown in Fig. 2, shorter periods of incubation with oxysterols were required to achieve 50% inhibition of [^{125}I]LDL uptake and degradation by all cultured cell lines. These results show that oxysterols are more potent and faster acting than cholesterol itself in modulating this process involved in cellular cholesterol homeostasis.

25-Hydroxycholesterol behaved as 27-hydroxycholesterol in inhibiting LDL receptor pathway in extra-hepatic cells, but was six times more potent than 27-hydroxycholesterol in hepatic cells, as inferred from IC_{50} values. The inability of low concentrations of 27-OH-C to interfere with LDL uptake and degradation could be explained by the observation that oxysterols can be metabolized by 7α-hydroxylase to form bile acids [22,29]. It can be speculated that 7α-hydroxylase maintains the expression of LDL receptors by metabolizing (i.e. inactivating) oxysterol repressor(s) [43]. Since Hep G2 cells are able to synthesize bile acids [44,45], and since 27-OH-C is a physiological bile acid precursor [22,44], part of this exogenously administered oxysterol can be utilized for this metabolic pathway and therefore it may not be completely available for regulating LDL receptors. This difference between the two oxysterols is in favor of a role, if any, of 27-OH-C in the regulation of cholesterol metabolism in extra-hepatic tissues; it has been, in fact, recently proposed that 27-OH-C is an intacrine regulator of sterol metabolism in ovarian cells [26]. A role for 27-OH-C as an effective modulator of cholesterol homeostasis in peripheral cells is consistent with the fact that the abundance of mRNA for 27-hydroxylase parallels the cholesterol biosynthetic capacity in most tissues [27].

The shorter periods of incubation with oxysterols required to achieve 50% inhibition of LDL uptake and

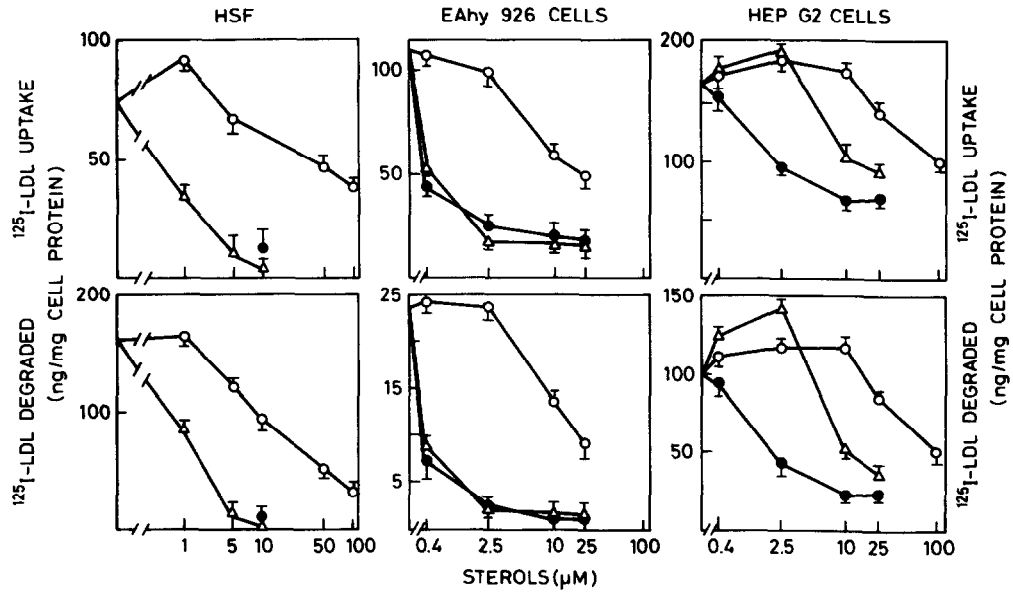

Fig. 1. Effect of increasing concentrations of sterols on [^{125}I]LDL uptake and degradation by human hepatic (Hep G2) and extrahepatic (HSF; EAhy 926) cell lines. Cells were preincubated for 24 h at 37°C in MEM containing 5% LPDS in the presence of the indicated concentrations of sterols [^{125}I]LDL (7.5 μg/ml) were then added and the incubation continued for further 5 h at 37°C. Uptake and degradation of [^{125}I]LDL were determined as described in section 2. Each experimental point represents the mean ± SD of three determinations. Cholesterol (○), 25-OH-C (●), 27-OH-C (△).

degradation could reflect a different rate of diffusion into the cell. In fact cholesterol has to equilibrate with a large membrane pool (about 90% of total cellular cholesterol) and must increase the cellular free cholesterol content before eliciting its effect on the expression of the genes regulating the synthesis of both LDL-receptor and HMG-CoA reductase [1]. Moreover, cholesterol is less polar than its oxygenated derivatives and it has been recently reported [21] than an analogue of 27-hydroxycholesterol, 26-aminocholesterol, that has the same polarity of 27-hydroxycholesterol, possesses a similar ability in regulating cellular cholesterol homeostasis. On the contrary another analogue, 26-thiacholesterol, which is less polar than 27-hydroxycholesterol and 26-aminocholesterol, is less effective in suppressing the expression of LDL-receptor [21].

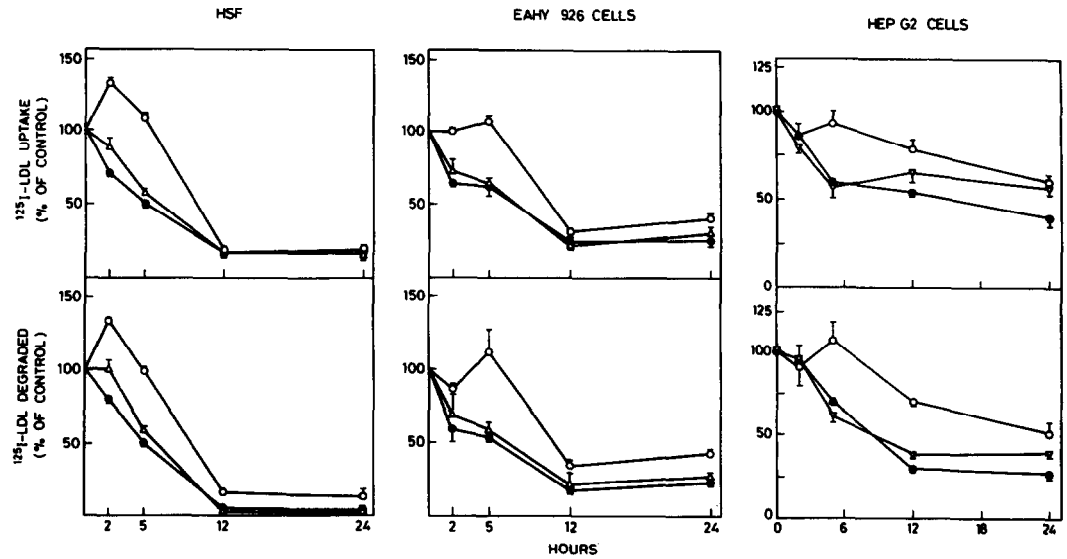

Fig. 2. Time-dependent effect of sterols on [^{125}I]LDL uptake and degradation by HSF, EAhy 926 and Hep G2 cells. Cells were incubated for 24 h at 37°C in MEM containing 5% LPDS; sterols were then added and the incubation continued for the indicated times. A fixed concentration of [^{125}I]LDL (7.5 μg/ml) was added and after 5 h the [^{125}I]LDL uptake and degradation were determined as described in section 2. Each experimental point represents the mean ± S.D. of three determinations. Cholesterol 100 μM (○), 25-OH-C 10 μM (●), 27-OH-C 10 μM (△).

These results suggest that sterol polarity could be more important than the presence of an oxygenated function on the molecule.

It should be mentioned that LDL-derived cholesterol exerts its effect before any increase in the overall cellular cholesterol content is detected [1]. This high physiological potency of LDL-derived cholesterol could be attributed to a carrier protein that binds cholesterol when it emerges from the lysosome and escorts it to regulatory sites. Alternatively, lysosomally liberated cholesterol could be converted into more active oxysterols [2]. A protein that could mediate the regulatory action of oxysterols has been purified [10,11] and its cDNA clones [12], although its metabolic role remains to be defined. Recently, Taylor [46] has shown that oxysterol potencies in the regulation of the degradation and the repression of HMG-CoA reductase were correlated with the relative affinities of oxysterols for an oxysterol binding protein, suggesting that this receptor is the element involved in both these regulatory pathways.

In summary our data indiate that oxysterols are more efficient than cholesterol, in terms of potency and time, in inhibiting the receptor mediated LDL pathway in human hepatic and extrahepatic cell lines. The effect of 27-OH-C occurs in extrahepatic cells at concentrations similar to those observed in human serum (0.2–0.6 μM) [30], supporting a role of this physiologcal oxysterol in the regulation of cellular cholesterol homeostasis in extrahepatic tissues.

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