

Distribution of exogenous 25-hydroxycholesterol in Hep G2 cells between two different pools

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Abstract Binding of [26,27-³H]25-hydroxycholesterol (25HC) to human hepatoma Hep G2 cells was saturated within 120 min. Two intracellular pools of 25HC were identified in a pulse-chase experiment: (i) an exchangeable pool which was in dynamic equilibrium with 25HC in the medium ($t_{1/2}$ of reversible exchange 15 min) and (ii) an unexchangeable pool which remained in cells during incubation in medium containing LPDS. 25HC from the exchangeable pool inhibits cholesterol biosynthesis, decreases the HMG CoA reductase mRNA level and stimulates cholesterol acylation. 25HC from the unexchangeable pool was partially bound to cytosolic proteins and apparently utilized for metabolic transformation. Incubation of Hep G2 cells with [26,27-³H]25HC in the presence of a 30-fold molar excess of 3 β -hydroxy-5 α -cholest-8(14)-en-15-one was found to cause (i) 2-fold decrease in the binding of [26,27-³H]25HC to cytosolic proteins (sedimentation constant of radioactive complex was 4–5 S) and (ii) the 35% inhibition of 25HC transformation to polar metabolites.

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Key words: Oxysterol; 25-Hydroxycholesterol; Sterol metabolism; Hep G2 cell

1. Introduction

Side chain oxygenated sterols are known to have a broad spectrum of biological activity in vivo and in cultured mammalian cells [1–4]. Endogenous 25-hydroxycholesterol (25HC) either synthesized in cell via the isoprenoid pathway [5] or formed in liver cells through oxidation of dietary cholesterol [6,7] regulates cellular cholesterol homeostasis by transcriptional control of sterol sensitive genes and stimulation of ACAT activity [8–14]. Exogenous 25HC added to cultured cells decreases the level of HMG CoA reductase and LDL receptor mRNAs, inhibits cholesterol biosynthesis, stimulates sterol esterification, affects the biosynthesis of triacylglycerols and phospholipids, modifies the cellular membrane structure,

suppresses the biosynthesis of protein and DNA, inhibits cell growth, causes apoptosis and cell damage [11–26].

Biological effects of exogenous 25HC and other oxysterols in cultured cells have been generally characterized only in terms of their concentrations in culture medium. However, studies of localization and traffic oxysterols in cultured cells are also important. These studies are necessary for the understanding of biological activity of oxysterols at the molecular level and for elucidation of cellular mechanisms underlying oxysterols inactivation and removal.

The binding of 25HC to cultured cells was shown to be rapid and saturable [27–31]. Metabolic transformation of 25HC depends on the cell type. In liver cells 25HC is transformed to normal bile acids [2,7,15,32] and undergoes ACAT-dependent 3-acylation with fatty acid residues [28]. Substantial 3-acylation of 25HC was found in peritoneal macrophages [27]. Slow 7 α -hydroxylation of 25HC by the enzyme differing from liver 7 α -hydroxylase was shown in human fibroblasts [33,34]. Transformation of 25HC to pregnenolon was demonstrated in mouse L fibroblasts [35].

Substantial proportion of exogenous 25HC in cultured cells was found in membranes, oxysterol being able to dissociate into media containing suitable acceptors [19,27]. The unidirectional flux of 25HC from plasma membrane to the membrane of endoplasmic reticulum in FuA5 rat hepatoma cells was found to be 2000 faster than the flux of cholesterol under the same conditions [28]. The binding of 25HC to the specific cytosol protein(s) OSBP was demonstrated in various cell types [11,29–31,36], however the role of OSBP in sterol metabolism and transport is not well established.

The present work was undertaken to study the traffic of exogenous 25HC in human hepatoma Hep G2 cells, the cellular model mostly utilized for the investigation of lipid metabolism in the human liver [37]. We demonstrate that the distribution of radiolabeled 25HC within Hep G2 cells can be represented in terms of two pools of 25HC differing in the respect of their exchange with 25HC in the media. The exchangeable pool of exogenous 25HC in Hep G2 is suggested to play the principal role in the regulation of sterol metabolism, while the unexchangeable pool is utilized for its metabolic transformations. The role of cytosolic proteins as specific carriers associated with transformation oxysterols to polar metabolites is discussed.

2. Materials and methods

25-Hydroxycholesterol, EDTA, Tris-(hydroxymethylamino)methane (Tris), ATP, glycylglycine, dithiothreitol and PBS were purchased from Sigma, Triton X-100 from Serva, oleic acid and organic sol-

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Abbreviations: 25HC, 25-hydroxycholesterol; [³H]25HC, [26,27-³H]25-hydroxycholesterol; 25HCE, 3 β -(25-hydroxycholesteryl) esters; PP, radioactive polar products formed from [26,27-³H]25-hydroxycholesterol by C-17 side chain scission; ACAT, acyl CoA:cholesterol acyltransferase; HMG CoA reductase, 3-hydroxymethyl-3-glutaryl CoA reductase; LDL, low density lipoprotein; OSBP, oxysterol binding protein; LPDS, lipoprotein deficient serum; FCS, fetal calf serum; PBS, phosphate buffered saline; RT-PCR, reverse transcription-polymerase chain reaction

vents: chloroform, hexane, methanol, ethanol, 2-propanol, toluene, ethyl acetate and acetic acid (HPLC grade) from Merck, [26,27-³H]25-hydroxycholesterol from New England Nuclear (NEN Research Products, Du Pont), [¹⁴C]acetic acid and [¹⁴C]oleic acid from Amersham. FCS, OptiMEM and F12 media were obtained from Gibco BRL, glutamin from 'ICN Biomedicals', culture plastics from Costar.

LPDS ($d > 1.23$ g/ml) was isolated from blood plasma of a healthy volunteer as described [38], followed by heating for 10 min at 60°C (for LCAT inactivation) and dialysis against PBS. 3 β -Hydroxy-5 α -cholest-8(14)-en-15-one (15-ketosterol) was synthesized according to Wilson et al. [39]. 3 β -(9-*cis*-Octadecenoyloxy)-cholest-5-en-25-ol was prepared from 25HC according to improved procedure of acylation [40] and isolated by TLC in 56% yield.

2.1. Hep G2 cells

Hep G2 cells obtained from European Cell Culture Collection (ECACC, Salisbury) were cultured in OptiMEM-F12 medium (1:1) with 10% FCS at 37°C in the atmosphere of 5% CO₂. Before the experiments the cells seeded in 35 mm dishes, or in 24-well plates, or in 96-well plates were preincubated for 24 h in the same medium containing 10% LPDS instead of FCS. All experiments were carried out in the media containing 10% LPDS.

Oxysterols were added to Hep G2 cells in ethanol solution. The final ethanol concentration in culture medium was 0.4%; in control incubations the concentration of ethanol was the same.

The toxicity of 25HC and 15-ketosterol for Hep G2 cells was checked by MST cytotoxicity assay using 'Promega Cell Titer Kit' according to the manufacturer's protocol. There were no cytotoxic effects of either 25HC at a concentration of 10 μ M or 15-ketosterol at a concentration of 30 μ M during 24 h incubation.

2.2. Binding of [³H]25HC to Hep G2 cells

Binding of [³H]25HC to Hep G2 cells was measured after incubation of cells during indicated time periods with [³H]25HC (6×10^5 cpm/ml) and non-labeled 25HC at a concentration of 10 μ M or 1 μ M (pulse); or in the absence of [³H]25HC (chase). Cells were rinsed with PBS at 4°C, treated 2 \times with 0.5 ml solubilising buffer [31] containing 1% Triton X-100, 1 mM ATP, 25 mM glycylglycine, 4 mM EDTA, 15 mM MgSO₄, 15 mM KH₂PO₄, 1 mM dithiothreitol (pH 7.8), then lipids were extracted with chloroform:methanol mixture (2:1). Chloroform phase was dried under nitrogen, radioactive products were separated by TLC in a toluene-EtOAc (3:2) system in the presence of non-radioactive 25HC. Radioactive zones were scrapped off and used for quantitation in liquid scintillator in a 'Tracor Instrument' radioactivity counter. All measurements were carried out in quadruplicates. Aliquots from aqueous phase were used for quantitation of polar radioactive products (PP) and determination of protein concentration [41].

2.3. Quantitation of radioactive 25HC, 25HCE and PP

Quantitation of radioactive 25HC, 25HCE and PP was performed in Hep G2 cells preincubated for 14 h with [³H]25HC (10^6 cpm/ml) and 25HC (1 μ M or 10 μ M) either in the absence or in the presence of 30 μ M 15-ketosterol. The cells were then incubated for 2 h, 2 \times 2 h and 3 \times 2 h in 0.5 ml of the medium containing 10% LPDS or in the same medium containing 30 μ M 15-ketosterol. The cells were treated with solubilising buffer as indicated above. Each cellular extract and each medium sample was separately treated with chloroform:methanol (2:1) mixture. Radioactivity of PP was measured in aqueous phases; chloroform phases were dried under nitrogen, radioactive products were analyzed by TLC in a toluene-EtOAc (3:2) system in the presence of non-radioactive standards: 25HC and 3 β -(9-*cis*-octadecenoyloxy)-cholest-5-en-25-ol. Radioactivity was measured as indicated above.

2.4. The rate of cholesterol biosynthesis and cholesteryl esters biosynthesis

The rate of cholesterol biosynthesis and cholesteryl esters biosynthesis in Hep G2 cells was measured by incorporation of [¹⁴C]acetate in cholesterol and cholesteryl esters [42] and by incorporation of [¹⁴C]oleate in cholesteryl esters [42].

Before addition of [¹⁴C]acetate or [¹⁴C]oleate, cells were preincubated: (1) in medium containing 10% LPDS for 3 h (control); (2) in the same medium plus 10 μ M 25HC for 3 h; (3) in the same

medium plus 10 μ M 25HC for 3 h followed by incubation in medium without 25HC for 3 h. The incubations were continued for 3 h more with [¹⁴C]acetate (5 μ Ci/ml) or with [¹⁴C]oleate (0.5 μ Ci/ml) for cell samples (1) and (3); or with those radioactive compounds plus 10 μ M 25HC for cell sample (2).

Cells were rinsed with PBS at 4°C, lipids were extracted by hexane:iPrOH (3:2, v/v) and separated by TLC in a hexane:diethyl ether:acetic acid (85:14:1). Incorporation of radioactivity in cholesterol, cholesteryl esters, triglycerides and free fatty acids was quantitated as above and referred to 1 mg of cell protein. All measurements were carried out in triplicates.

2.5. HMG CoA reductase mRNA

HMG CoA reductase mRNA level was measured in Hep G2 cells preincubated: (1) in medium containing 10% LPDS for 3 h (control); (2) in the same medium plus 10 μ M 25HC for 3 h; (3) in the same medium plus 10 μ M 25HC for 3 h followed by incubation in medium without 25HC for 4 h; (4) in the same medium plus 10 μ M 25HC for 7 h.

Total RNA from Hep G2 cells was isolated by acid guanidine isothiocyanate [43]. The quantitative RT-PCR was performed by the method based on the intraspecies polymorphism [44], using the total RNA from mouse liver as an internal standard. The mixture of mouse RNA (1 μ g) and RNA from Hep G2 (1 μ g) was subjected to RT as described [44]. The following primers were used for PCR amplification: 5'-ATAGGTGGCTACAACGCCC-3' (direct), and 5'-ATGTTAGTCCTTAAGAACCCAATGC-3' (reverse) corresponding to 244–262 and 706–730 nucleotides of mouse HMG CoA reductase cDNA sequence. After digestion of PCR products with *Hinf*I restriction endonuclease, the fragments were separated in a 2% agarose gel and stained with ethidium bromide. Quantitation of each band was performed using an 'Alpha Innotech's' digital imaging system. The quantity of HMG CoA reductase mRNA was normalized to β -actin mRNA content in the same sample.

2.6. Binding of [³H]25HC to cytosolic proteins

Hep G2 cells in 35 mm dishes were incubated for 3 h with 2 ml medium containing [³H]25HC (1×10^6 cpm/ml), 1 μ M 25HC and 10% LPDS or in the same medium in the presence of 30 μ M 15-ketosterol.

Cytosolic proteins were isolated according to [30] with minor modifications. The cells were rinsed twice with PBS at 4°C and frozen at –70°C. Then the cells were scrapped with a rubber policeman, homogenized in 0.5 ml of the buffer (50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) and centrifuged at 4°C and 40000 rpm in a Beckman L5-55 ultracentrifuge (LP 42 Ti rotor) for 40 min. The supernatants containing [³H]25HC were applied on a performed 5–20% sucrose gradient. Ultracentrifugation was carried out at 4°C and 40000 rpm in a Beckman L5-55 ultracentrifuge (SW 40 Ti rotor) for 20 h.

After centrifugation, 0.8 ml fractions were picked up and used for quantitation of radioactivity and measurement of sucrose concentration by refractometry. Sedimentation constants of related radioactive fractions were estimated by comparison with standards (human serum albumin, $S_w^{20} = 4.3$ S and γ -globulin, $S_w^{20} = 7.0$ S) in an isokinetic sucrose linear gradient, or calculated by the general method [45] with reported modifications [46].

3. Results

The rate of 25HC exchange between cultured cells and medium is known to be dependent on the medium composition and cellular metabolism peculiarities [19,27]. Since all experiments in the present study were carried out in the presence of 10% LPDS, the binding of radiolabeled 25HC to Hep G2 cells was studied at constant concentrations (1 μ M or 10 μ M) of non-labeled 25HC in the medium. Hep G2 cells were relatively stable in respect to toxic effects of 25HC: a 24 h incubation of cells with 25HC at a concentration of 10 μ M did not cause cell damage, as demonstrated by MST assay.

The binding of [³H]25HC to Hep G2 cells was studied in a pulse-chase experiment (Fig. 1). The content of radioactivity in cells was saturated within 120 min and was higher at 10 μ M

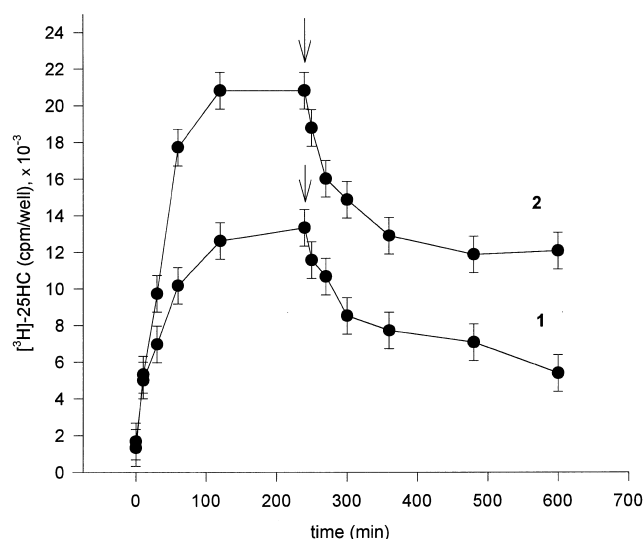


Fig. 1. Binding of [^3H]25HC to Hep G2 cells at a concentrations of 25HC of 1 μM (1) and 10 μM (2). For details see Section 2. Protein concentration per well was 0.2 mg. The arrow indicates the start of chase.

25HC compared with those at 1 μM . Preincubation of Hep G2 cells for 4 h with non-radioactive 10 μM 25HC affected neither the rate of binding of radioactivity to cells nor the content of radioactivity within cells at the saturation. Preincubation of Hep G2 cells with 30 μM 5 α -cholest-8(14)-en-15-on-3 β -ol (15-ketosterol, the known synthetic inhibitor of sterol biosynthesis [47]) also did not affect the binding of [^3H]25HC to cells. The binding curves for cells preincubated with 25HC or with 15-ketosterol were the same as shown in Fig. 1.

Further incubation of Hep G2 cells prelabeled with [^3H]25HC in medium containing non-radioactive 25HC at the same concentration (chase) caused the efflux of 50–60% of radioactivity from the cells within 120–150 min, the rate of binding [^3H]25HC being equal to the rate of efflux. Substantial part of [^3H]25HC (40–50%) remained within the cells. Not less than 88% of the cell radioactivity during a 6 h period was found in 25HC fraction. Therefore, [^3H]25HC bound to Hep

G2 cells could be divided into two pools which differ in respect to the rate of their exchange with 25HC in the media. The $t_{1/2}$ value of equilibrium exchange between 25HC in media and 25HC in exchangeable pool was calculated to be near 15 min.

Exogenous 25HC which belongs to these different pools was assumed to have different effects on sterol metabolism. To check this assumption we compared the well known effects of 25HC on the levels of HMG CoA reductase mRNA (Fig. 2A), cholesterol biosynthesis (Fig. 2B) and cholesteryl esters biosynthesis (Fig. 2C and D) in Hep G2 cells incubated with 10 μM 25HC for 3 h with those if cells were subsequently chased for 3 h in medium without 25HC. Obviously, 25HC which belongs to exchangeable pool must efflux from the cells during the chase.

Comparison of Fig. 2A and B shows that both HMG CoA reductase mRNA level and cholesterol biosynthesis rate are effectively inhibited by 25HC. These effects occur during a 3 h incubation. There were no significant differences depending on the incubation of prelabeled cells with LPDS. Incorporation of [^{14}C]acetate in triacylglycerols, phospholipids and free fatty acids did not depend on the presence of 25HC in our experiments.

Although the inhibition of HMG CoA reductase activity decreased the incorporation of [^{14}C]acetate in cholesterol, the total incorporation of radioactivity in cholesteryl esters in the presence of 25HC was increased (2, Fig. 2C). This effect was evidently due to the stimulation of ACAT activity by 25HC [12,14]. Subsequent incubation of cells in medium without 25HC (chase) abolished this effect (3, Fig. 2C). The relative rates of cholesteryl esters synthesis from [^{14}C]oleate depended on the incubation of prelabeled cells with LPDS (Fig. 2D). These data indicated that 25HC which belongs to exchangeable pool takes the principal part in stimulation of sterol esterification.

Prolong incubation of Hep G2 cells with [^3H]25HC led to the appearance of radioactive metabolites. Quantitation of radioactive products was performed in Hep G2 cells preincubated with [^3H]25HC for 14 h. Radioactive products were analyzed by TLC in a toluene-EtOAc (3:2) system in the presence of related synthetic standards. Besides 25HC (R_f

Table 1

The content of radioactive products (%) in prelabeled Hep G2 cells and in medium during incubation^a

Time of chase	Radioactive compound	Content within cells	Content in medium ^b
0	Total	100	—
	25HC	69 (± 4)	—
	25HCE	22 (± 3)	—
	PP	4 (± 2)	—
2	Total	71 (± 6)	33 (± 5)
	25HC	45 (± 4)	21 (± 3)
	25HCE	23 (± 4)	2
	PP	< 2	11 (± 5)
4	Total	57 (± 6)	48 (± 6)
	25HC	22 (± 4)	33 (± 4)
	25HCE	27 (± 7)	3 (± 2)
	PP	< 2	14 (± 4)
6	Total	46 (± 5)	56 (± 6)
	25HC	18 (± 3)	31 (± 2)
	25HCE	24 (± 4)	4 (± 2)
	PP	< 2	22 (± 5)

^aQuantitation of radioactive products was performed as described in Section 2. The mean values of four independent experiments are given. The total radioactivity of prelabeled cells in each experiment was assumed as 100%.

^bThe content of radioactive products after 4 and 6 h incubation in medium was obtained as a sum of two and three medium samples for each well, respectively.

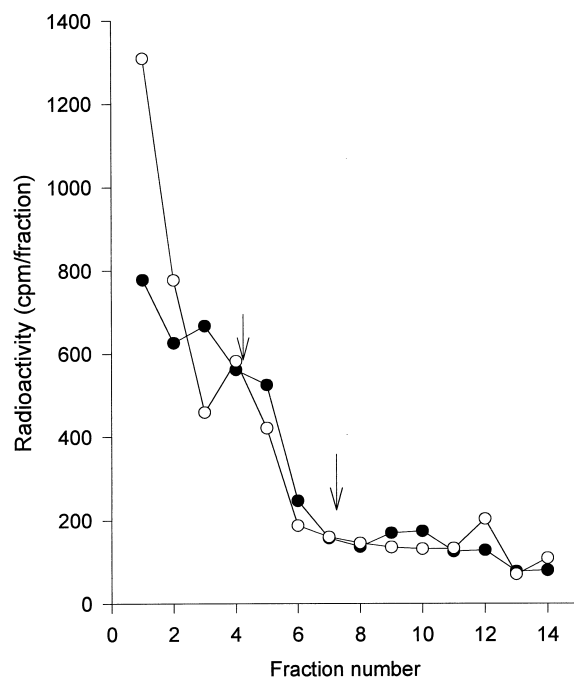


Fig. 4. Binding of [3 H]25HC to cytosolic fraction from Hep G2 cells. Sucrose gradient ultracentrifugation of cytosol isolated from Hep G2 cells prelabeled with [3 H]25HC in the absence (filled circles) and in the presence (open circles) of 15-ketosterol; arrows indicate positions of standards: human serum albumin and γ -globulin; the volume of each fraction was 0.6 ml; details are given in Section 2.

under the conditions reported previously [30] for isolation of the 25HC-OSBP complex (7.6 S) from human leukemic T-cells. In our experiments cytosolic fraction contained 12% of radioactivity bound to cells in the absence and 18% in the presence of 15-ketosterol. [3 H]25HC was found to be the only radioactive compound in the cytosolic fraction. The profile of the ultracentrifugation of radioactive cytosol in a 5–20% sucrose gradient is shown in Fig. 4. [3 H]25HC was found both in fraction 1 (apparently non-bound to proteins) and in fractions 3–5 with sedimentation coefficient 4–5 S. The binding of 25HC to cytosolic proteins with the same sedimentation coefficient was shown earlier [49] in mouse fibroblasts, the binding of [3 H]25HC to fraction 7.6 S in our experiments was not observed. The presence of 15-ketosterol increased the total content of [3 H]25HC in the cytosol for approximately 50–60% with simultaneous 2-fold decreasing of [3 H]25HC binding to the fraction 4–5 S. These data suggest that part of [3 H]25HC bound to Hep G2 cells exists in a complex with cytosolic proteins and 15-ketosterol affects the concentration of this complex.

Taken together, the data shown in Figs. 3 and 4 suggest that the binding of oxysterols to cytosolic proteins is important for transformation of oxysterols to polar metabolites.

4. Discussion

About 20 years ago Kandutch and coworkers put forward a hypothesis that cholesterol biosynthesis in cells is normally regulated by endogenously synthesized oxysterols [50]. Intracellular concentration of oxysterols is reduced by incubation of cells in media containing suitable acceptors for oxysterols [19,27]. The decrease in the intracellular oxysterol content

caused by incubation of cells in LPDS activates HMG CoA reductase and LDL receptor with a simultaneous elevation of the corresponding mRNAs [3,8–11]. On the other hand, the intracellular binding of oxysterols to specific cytosolic proteins is well documented [11,29–31,36,48,49].

We have suggested that some information regarding intracellular transport of endogenous oxysterols and their role in the sterol metabolism regulation could be obtained from studies of exogenous oxysterol traffic. The results of the present study demonstrate that exogenous 25HC in Hep G2 cells is distributed between two pools. A scheme illustrating the traffic of exogenous 25HC in Hep G2 cells was proposed (Scheme 1).

Exogenous 25HC rapidly and reversibly binds to Hep G2 cells and probably joins the endogenous oxysterols (exchangeable pool). 25HC which belongs to this pool does not bind to OSBP, since neither preincubation with 25HC nor the excess of 15-ketosterol affects the binding of radiolabeled 25HC to the cells during a short incubation. The exchangeable pool of 25HC was found to be in dynamic equilibrium with 25HC in the culture medium and to stimulate sterol esterification. Inhibition of cholesterol biosynthesis and HMG CoA reductase activity by 25HC occurs after a short incubation. Therefore, we assumed that this exchangeable pool of 25HC plays the principal role in the regulation of the sterol metabolism, interacting with nuclear receptors and mediating the known cascade regulation of sterol sensitive genes [51–53].

Twelve–eighteen percents of 25HC from unexchangeable pool was found in cytosolic fraction. The presence of 15-ketosterol reduces the binding of 25HC to cytosolic proteins with a simultaneous decrease in the oxidative transformation of 25HC to polar metabolites. Therefore, we have suggested that the specific cytosolic proteins serve as carriers that participate in inactivation and degradation of the oxysterol, rather than mediate their regulatory effects.

It was reported that resistance of some cell lines to the toxic effect of 25HC correlates with the intracellular level of OSBP [30]. (3 α ,4 α ,5 α)-4-(2-Propenylcholestan-3 β -ol) (LY295427) was found to stimulate the binding of 25HC to OSBP in CHO-K1 cells and to cause the binding of 25HC to 170 kDa cytosolic protein from hamster liver [31]. Enhanced binding of 25HC to cytosolic proteins was found to stimulate LDL receptor activity [54]. Recent studies in transformed CHO-K1 cells, which express a wild-type rabbit OSBP [55], demonstrated that overexpression of OSBP results in 50% decrease in the cholesteryl esters synthesis stimulated by 25HC and a 50% elevation of mRNA levels for three sterol sensitive genes (HMG CoA reductase, HMG CoA synthase and LDL receptor). 15-Ketosterol, which is as effective as 25HC in the ability to inhibit HMG CoA reductase activity in Hep G2 cells [56], nevertheless was 10-fold less potent in decreasing the corresponding mRNA level in the same cells [57].

All these data can be explained suggesting that OSBP and related cytosolic proteins are capable to bind regulatory oxysterols within cells with the formation of inactive complex, which is utilized for transformation of oxysterols to polar metabolites. The concentration of this complex as well as the concentration of free regulatory oxysterols, obviously, must depend on the presence of other compounds with the affinity for cytosolic proteins.

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