Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 182-189

CORRELATION AMONG OXYSTEROL POTENCIES IN THE REGULATION OF THE DEGRADATION OF 3-HYDROXY-3-METHYLGLUTARYL COA REDUCTASE, THE REPRESSION OF 3-HYDROXY-3-METHYLGLUTARYL COA SYNTHASE AND AFFINITIES FOR THE OXYSTEROL RECEPTOR[1]

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Received May 21, 1992

SUMMARY: 25-Hydroxycholesterol regulates cholesterol biosynthesis by two mechanisms: repression of the transcription of the genes for several cholesterogenic enzymes and acceleration of the degradation of the enzyme 3-hydroxy-3-methylglutaryl CoA reductase. In the present work the structural features which govern oxysterol potency were determined separately for each regulatory mechanism. Regulation of degradation was tested using a 3-hydroxy-3-methylglutaryl CoA reductase-β-galactosidase fusion protein. Repression of enzyme synthesis was tested by measuring 3-hydroxy-3-methylglutaryl CoA synthase activity since this protein is not regulated by a degradative mechanism. Oxysterol activities were highly correlated between the two assays (R=.959) demonstrating that the degradative and repressor mechanisms share an element which determines oxysterol regulatory potency. Correlation of these results with previous data for the affinity of these oxysterols for the oxysterol receptor suggests that the receptor is the element involved in both these regulatory pathways.

Cholesterol biosynthesis in mammalian cells is inhibited by certain oxygenated derivatives of cholesterol and lanosterol (oxysterols) as a result of their repression of several enzymes of the cholesterol biosynthetic pathway. One of the most potent oxysterols, 25-hydroxycholesterol, represses cholesterogenic enzymes by two mechanisms. One of these is the transcriptional regulation of the genes for HMGR (1), HMGS (2), and the LDL receptor (3). This has been demonstrated using chimeric constructs combining the promotor regions of these genes with reporter genes (3-10) and mutational analysis has revealed the existence of a similar sequence motif in each promotor, termed the sterol regulatory element, which is required for oxysterol regulation (reviewed in (11)).

[1] This work was supported by Grant CA 02758 awarded by the National Cancer Institute, Department of Health, Education and Welfare.

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The abbreviations used are: HMGal, 3-hydroxy-3-methylglutaryl CoA reductase-β-galactosidase fusion protein; HMGS, 3-hydroxy-3-methylglutaryl CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; LDL, low density lipoprotein; CHO, Chinese hamster ovary cell line.

0006-291X/92 $4.00

The second regulatory effect of 25-hydroxycholesterol is to increase the rate of degradation of HMGR. HMGR is a short half-life protein and in cell cultures its rate of degradation can be increased several fold by the addition of 25-hydroxycholesterol (12, 13). The HMGR enzyme consists of two domains: the carboxy-terminal two-thirds is a catalytic domain and it is anchored to the cytoplasmic face of the endoplasmic reticulum by a membrane spanning domain at the amino-terminus of the protein (14). Degradative control of HMGR activity is lost when the membrane domain is removed and the catalytic domain is no longer anchored to the endoplasmic reticulum (15, 16). Simoni and coworkers have shown that when the membrane domain is fused to the amino-terminus of ß-galactosidase, the addition of LDL or mevalonic acid can accelerate the rate of degradation of the chimeric protein in a fashion similar to their effect upon the intact HMGR protein (17-20).

In previous work the relationship between oxysterol structure and repressor potency was investigated by determining the concentrations required to repress HMGR activity by 50% in mouse L cells for a large number of oxysterols (21-24). In this assay cells were incubated for five hours with a range of oxysterol concentrations, after which HMGR enzyme activity in cell homogenates was measured. It has been established that oxysterols with the greatest repressor activity have a hydroxyl or keto substituent at C-25, C-26, or C-15 (half maximal repressor activity at ~10^{-8}M), while substitution at various other sites results in compounds with a range of lower activities. In addition, both the 3ß-hydroxyl group and the full length side chain of cholesterol are required for high activity and the stereochemistry of the second oxygen function and of the sterol ring system also govern repressor potency (21).

A shortcoming of the L cell HMGR repression assay as a measure of oxysterol potency is that the level of HMGR enzyme activity is a composite of the effects of the oxysterol on both transcription and degradation and it is not known whether oxysterols produce the two effects by similar or different mechanisms. In the present work the structural features which govern oxysterol potency was determined separately for each regulatory mechanism. Degradation was assayed using the HMGal Chinese hamster ovary (CHO) cell line containing the HMGR-ß-galactosidase fusion protein created in Simoni's laboratory. Repression of enzyme synthesis was assayed by measuring the repression of HMGS enzyme activity since this protein is not regulated by a degradative mechanism. The results were correlated with the relative affinities of the oxysterols for an oxysterol binding protein to assess the potential role of this protein in each mechanism.

EXPERIMENTAL PROCEDURES

Sterols. Cholesterol, 7-ketocholesterol and 20α-hydroxycholesterol were obtained from Sigma and 25-hydroxycholesterol from Steraloids. 3β-hydroxycholest-8(14)-en-15-one, lanost-8-ene-3β,32-diol, cholest-7-ene-3β,11α-diol, 3β-hydroxycholest-9(11)-en-12-one, and cholest-8(14)-en-15-one were gifts from George Schroepfer, Jr, Rice University. 24(S)-Hydroxycholesterol was a gift from Thomas Spencer, Dartmouth College, (25R)26-hydroxycholesterol was a gift from the late Henry Kircher and 1,25-dihydroxycholecalciferol was a gift from Hoffmann-La Roche, Inc. 26,27-Bisnorcholest-5-ene-3β,20-diol (25) and 25-hydroxycholest-4-en-3-one (21) were synthesized in our laboratory.

Assay for oxysterol-accelerated degradation of the HMGR-β-galactosidase fusion protein in CHO cells. The CHO-K1 cell line transfected with the HMGal fusion protein was generously

provided by Dr. Robert Simoni. The cells were grown as monolayers in Eagle's minimum essential medium supplemented with non-essential amino acids, 292 μg/ml glutamine, 250 μg/ml active geneticin (G418) and 4 mg/ml of delipidated fetal calf serum prepared by the method of Rothblat *et al* (26). In order to preserve expression of β-galactosidase a large number of cells were grown immediately upon receipt and aliquots frozen in liquid N_2. For each experiment a new aliquot was thawed and the cells used in the first or second passage. To begin an experiment the cells were suspended by trypsin treatment, washed with medium and resuspended at a concentration of 200,000 cells/ml. One half ml of this suspension was pipetted into each well of 24 well culture dishes (Costar) and incubated in a 37°C, 5% CO_2, humidified incubator for 48 h before the sterol solutions were added. A dilution series of each sterol was made in ethanol and 5 μl aliquots were added to each well, with control incubations receiving 5 μl of ethanol. The concentration range tested in the HMGal assay was based on each sterol's potency in the previously reported HMGR assay (21). After 3 h incubation the medium was poured off; each well was washed with 1 ml of phosphate buffered saline and drained thoroughly. The method for assaying HMGal degradation was adopted from Skalnik et al (18). The cells were permeabilized by shaking for 10 min at room temperature with 250 μl of 50 μg/ml digitonin in CSK buffer as described by Leonard and Chen (27). Then 1 ml of buffer Z (18) containing 1 mg/ml O-nitrophenyl galactoside was added to the digitonin solution in each well and incubation was continued for 3 h after which the reaction was stopped by the addition of 1 ml of the reaction volume to 0.5 ml of 1 M sodium carbonate. Activity was quantitated by measuring optical density at 420 nm.

Assay for oxysterol repression of HMGS in L cells. Mouse L cell fibroblasts (a subline of NCTC clone 929) were grown in spinner cultures in serum-free Waymouth's 752/1 medium as described (28). To begin an experiment cells (4 x 10^6) in 15 ml medium from the L cell spinner culture were pipetted into 75 cm^2 culture flasks and placed in a 37° C, 5% CO_2, humidified incubator. After 6 h the cells had attached and the medium was poured off and replaced with 13.5 ml of fresh medium. Twenty hours later sterols were added to the medium in 1.5 ml of a solution of 5% bovine serum albumin and 1% ethanol with 5 μg/ml α-tocopheral as an antioxidant as described (29). Control incubations received the vehicle without sterol. After incubation for 20 h, the cells were suspended in the medium with a rubber policeman and collected by centrifugation, washed once in 2.5 ml phosphate buffered saline and resuspended in 0.4 ml of PED buffer (20 mM Na phosphate, 0.1 mM EDTA, 0.5mM dithiothreitol, pH 7.2). The cells were dispersed and disrupted by three one sec bursts of sonication with a Bronson microprobe at the lowest energy setting and the sonicate centrifuged at 207,000 g for 22 min. Duplicate 80 μl aliquots of the supernatent were dialyzed *vs* PED buffer in a MEGA System Microdialyzer (Pierce) before assaying for protein and HMGS activity. Protein was determined by the Bradford reagent (Bio-Rad).

HMGS activity in the cytosolic supernatent fraction was assayed by the radiolabel method originated by Clinkenbeard et al (30) except that after heating at 95°C for 1.5 h with 0.4 ml of 6 N HCl to remove unreacted (^{14}C)acetyl-CoA, an additional 0.2 ml of 6 N HCl was added and heating was continued for another 1 h. This step gave a lower and more consistent background. Concentrations of the synthase reaction components were: 100 mM TrisHCl, pH 8, 5 mM $MgCl_2$, 600 μM acetyl CoA (Sigma, A-2056), 0.1 μCi [acetyl-1-^{14}C]acetyl-CoA (New England Nuclear, NEC-313), 50 μM acetoacetyl-CoA (Sigma, A-1625) and 100-200 μg cytosolic protein in a final volume of 200 μl. In addition, 50 nCi of DL-3-[methyl-^{3}H]hydroxy-3-methylglutaryl-CoA (New England Nuclear, NET-560) was added at the beginning of the reaction as an internal standard for product recovery. After incubation for 20 min at 30°C the reaction was terminated as described above. Radiolabel in a zero time control incubation was subtracted from experimental values.

Calculation of oxysterol repressor potency. The data for each assay was plotted as the percent of control activity *versus* the natural log of the sterol concentration in the medium. The concentration of the sterol which resulted in a 50% of the maximum response was determined by least squares analysis of the linear portion of the curve.

RESULTS AND DISCUSSION

Oxysterol regulated degradation of the HMGR-β-galactosidase fusion protein. The fusion protein HMGal, constructed in Robert Simoni's laboratory, consists of the membrane and

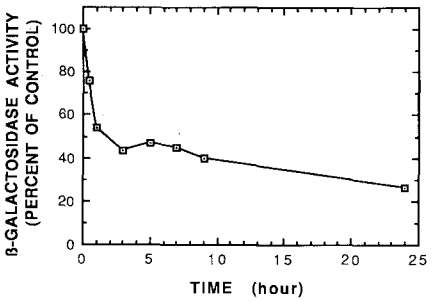


Fig. 1. Time course of 25-hydroxycholesterol-induced degradation of the HMGal fusion protein. CHO K1 cells carrying the HMGal fusion construct were grown in 24 well culture dishes and ß-galactosidase activity was assayed as described under "Experimental Procedures". 25-Hydroxycholesterol (5mM) was added in quadruplicate in a staggered so that all timepoints were assayed simultaneously.

cytoplasmic linker domains of HMGR coupled to the amino terminus of β-galactosidase (18). Transcription of the hybrid gene is driven by the SV40 early promoter which is not regulated by sterols (13). Simoni's group obtained transfectants of the construct in CHO-K1 cells by cotransfection of a plasmid carrying the neomycin-resistance gene. They have demonstrated using enzyme assays and immunoprecipitation that the degradation rate of the fusion protein is accelerated to the same extent as the native HMGR protein by treatment with LDL or mevalonic acid (17, 18). Figure 1 shows the time course for the decline in β-galactosidase activity of the HMGal protein in response to treatment with 5 μM (2 μg/ml) 25-hydroxycholesterol. Approximately 50% of the initial activity was lost during the first h after which the rate of decline slowed or stopped. The remaining activity may represent, at least in part, a new steady state of synthesis and degradation. In order to examine the effect of oxysterol structure on the initial fast rate of degradation subsequent incubations were for 3 h.

The fourteen sterols tested were chosen to encompass the general structural features found to be critical in determining repressor potency in the L cell HMGR assay. Five concentrations of each sterol were tested, spanning a fifty-fold concentration range, and each point was determined in duplicate in each of at least two separate experiments. The results are presented in Table 1. Cholesterol, recrystallized three times from ethanol, showed no activity in the HMGal degradation assay, demonstrating an absolute requirement for the second oxygen function. Certain features of the cholesterol molecule must be retained for the oxysterol to exhibit high activity. For example when the 3β-hydroxy function is removed from 15-ketocholesterol activity is much reduced. Similarly when the 5-ene-3β-hydroxy structure of 25-hydroxycholesterol is converted to a 4-ene-3-one structure activity is also greatly reduced. The full-length C-8 side chain of cholesterol is also important for activity as evidenced by the reduced activity of the 26,27-bisnor derivative of 20α-hydroxycholesterol. 1,25-dihydroxycholecalciferol, which has a disrupted B ring and the additional 1-hydroxy function, was completely inactive in the HMGal degradation assay. The position of the second oxygen function also modulated oxysterol potency. The relative activity order was 25-hydroxy, (25R)26-hydroxy>20-hydroxy, 15keto, 24(S)-hydroxy>7-keto, 11-

TABLE I
Oxysterol regulatory activity and relative affinity for the oxysterol receptor

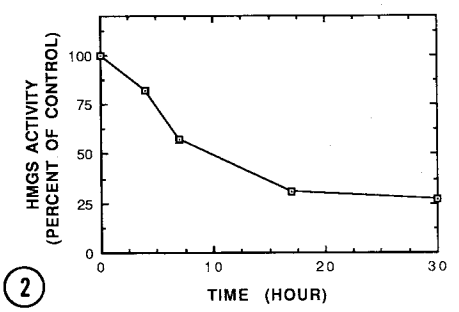
Sterol	HMGal degradation[a]	HMGS repression[a]	HMGR repression[a,b]	Oxysterol recep binding affinity[a]
Cholest-5-ene-3β,25-diol	0.10	0.33	0.17	0.03
(25*R*)-Cholest-5-ene-3β,26-diol	0.13	0.66	0.26	0.11
Cholest-5-ene-3β,20α-diol	0.34	0.46	0.30	0.06
Cholest-5-ene-3β,24(S)-diol	0.37	0.61	0.78	0.79
3β-Hydroxy-5α-cholest-8(14)-en-15-one	0.43	0.75	0.10	0.02
5α-Lanost-8-ene-3β,32-diol	0.58	-	0.07	0.05
26,27-Bisnorcholest-5-ene-3β,20α-diol	1.11	1.22	1.20	0.47
5α-Cholest-7-ene-3β,11α-diol	1.59	1.73	0.55	0.36
3β-Hydroxycholest-5-en-7-one	1.93	1.31	1.70	1.40
3β-Hydroxy-5α-cholest-9(11)-en-12-one	4.30	4.38	1.00	0.40
5α-Cholest-8(14)-en-15-one	7.29	-	3.00	1.80
25-Hydroxycholest-4-en-3-one	11.43	6.28	3.50	2.40
1,25-Dihydroxycholecalciferol	NR	-	NR	NR
Cholesterol	NR	NR	NR	NR

[a]The values given are the μM concentrations necessary for 50% response in each assay calculated as described und "Experimental Procedures." Sterols which gave no response at the highest concentration tested are indicated by "NR. Sterols not tested in the HMGS repression assay are indicated by a dash.
[b]Data taken from reference 21.

hydroxy>12-keto. A natural hydroxylated intermediate in the cholesterol biosynthetic pathway, 32-hydroxylanost-8-en-3β-ol, also showed considerable activity in the HMGal degradation assay.

Oxysterol regulation of HMGS. The HMGS enzyme is a soluble protein, lacking the membrane-spanning domain which is necessary and sufficient for the regulation of HMGR protein degradation. It has been shown that the HMGS protein is stable to 25-hydroxycholesterol treatment in 25-hydroxycholesterol-resistant cell lines which have lost oxysterol regulation of gene transcription but which retain degradative control of the HMGR protein (31). It has also been shown that treatment of HepG2 cells with LDL does not increase the rate of degradation of HMGS (32). Therefore the level of HMGS activity in cells can be used to assess the repressor potency of oxysterols independently of changes in protein degradation rates.

The relative potency of oxysterols in repressing HMGS activity was measured in mouse L cells. The HMGS assay, adapted from Clinkenbeard *et al* (30), was optimized in L cells with respect to pH, Mg^{++}, protein concentration and time (data not shown). Enzyme activity was enhanced ~20% by overnight dialysis attributable, at least in part, to a loss of HMG-CoA lyase activity (data not shown) as previously discovered (30). Recoveries of tracer amounts of (^{3}H)HMG-CoA, which was included as an internal standard at the beginning of the assay, averaged 75%. The time course of repression of HMGS activity by 5 mM 25-hydroxycholesterol is shown in Fig 2. Activity declined with an initial half-life of ~7 h until 27% of the activity remained. In order to maximize the response, subsequent incubations to test oxysterol activity were conducted for 20 h. Eleven of the sterols tested in the HMGal degradation assay were also tested in this assay (Table 1). The same general features were found to be important for activity i.e. the 3β-hydroxy group, an intact side chain, and a second oxygen function at particular locations.



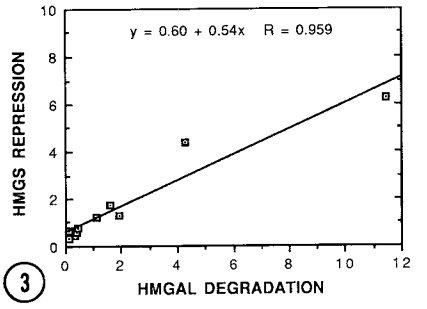


Fig. 2. Time course of 25-hydroxycholesterol-induced repression of HMGS activity in L cells. 25-Hydroxycholesterol (5mM) was added in a staggered fashion so that all timepoints were assayed simultaneously. The cells were grown in 75 cm^2 flasks and assayed for HMGS as described under "Experimental Procedures".

Fig. 3. Correlation between oxysterol-induced degradation of the HMGal fusion protein and oxysterol repression of HMGS. Data taken from Table I.

Correlations. The results from the HMGal degradation assay and the HMGS repression assay were compared to each other and to previous data for the activity of the same oxysterols in the repression of HMGR in L cells and for their relative binding affinities to the L cell oxysterol receptor. There was a very strong correlation between activity in the HMGal degradation assay and the HMGS repression assay (Fig 3) yielding a correlation coefficient of 0.959. In addition there was a strong correlation between these two assays and the previous data for the repression of HMGR in L cells (Fig 4; HMGal *vs* HMGR, 0.921; HMGS *vs* HMGR, 0.818). The most important conclusion that can be derived from these correlations is that the degradative and repressor control mechanisms share a central element which determines the relative regulatory potency of oxysterols. It also appears that the specificity of this functional element is evolutionarily conserved between the Chinese hamster ovary cells used in the HMGal degradation assay and the mouse L cells used in the HMGS and HMGR assays.

Correlation between the relative binding affinity of the oxysterol repressor for over 60 oxysterols and the potency of these oxysterols in repressing HMGR activity in L cells (0.84) has supported the argument that this protein may be a receptor which mediates at least part of the biological response (21, 22, 23, 24, 33). This correlation was very high (0.95) for the oxysterols selected for use in the present study. The relative binding affinity of the receptor also correlates with activity in the HMGal degradation assay and the HMGS repression assay (.874 and .720, respectively, Fig 5). Aside from the experimental error in the determination of the values for these parameters, less than perfect correlation would be expected in assays comparing a biological response in whole cells with a cell-free binding assay due to the different problems of oxysterol solubilization and interaction with other constituents in the assays. It should be noted that the correlation of the relative regulatory potencies between assays is valid despite differences in the molar concentrations required to achieve a regulatory effect in each assay. The differences in the molar concentrations required among the assays will be determined by the solubility, uptake and

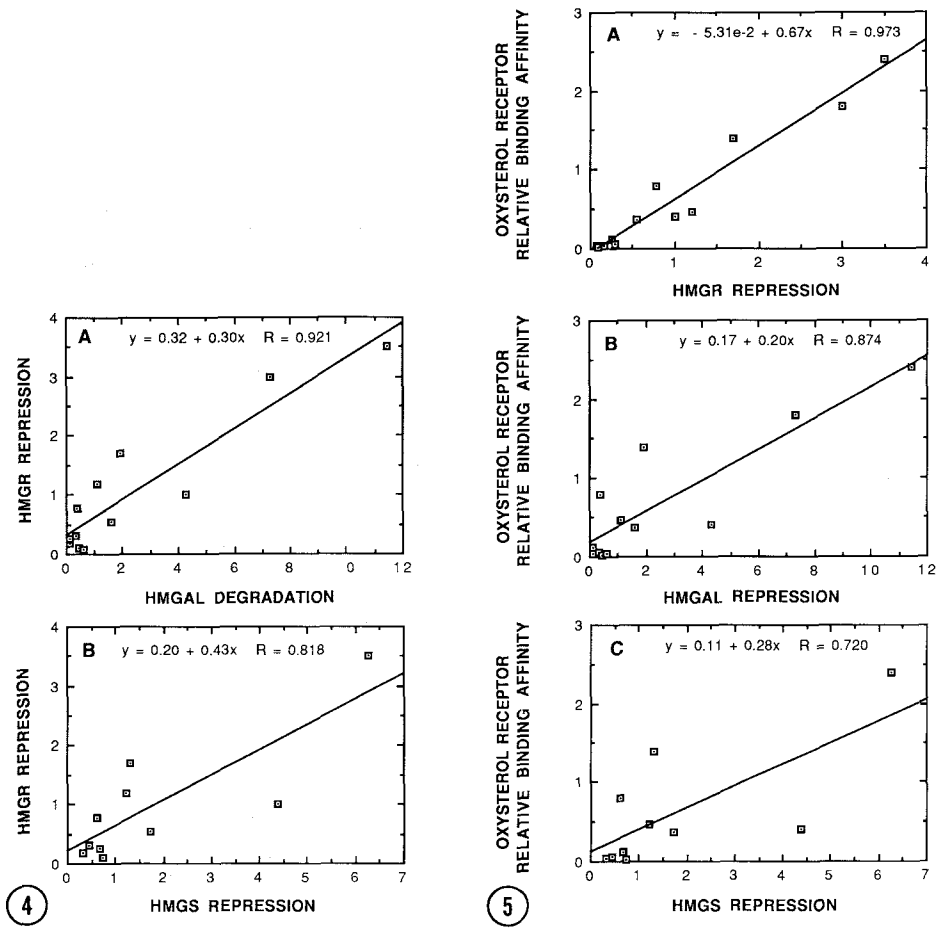


Fig. 4. Correlation between oxysterol repression of HMGR and (A) oxysterol-induced degradation of the HMGal fusion protein and (B) oxysterol repression of HMGS. Data taken from Table I.

Fig. 5. Correlation between oxysterol affinity for the oxysterol receptor and (A) oxysterol repression of HMGR, (B) oxysterol-induced repression of HMGal fusion protein and (C) oxysterol repression of HMGS. Data taken from Table I.

distribution of oxysterols under each set of conditions and most importantly by the point at which 50% repression is measured during the time course of the regulatory response. With these factors in mind, the data appear to support a hypothesis that the oxysterol receptor may be involved in both the degradative and transcriptional responses.

Acknowledgments - We thank Dr. Robert D. Simoni for the gift of the CHO cell line carrying the HMGal fusion protein.

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