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SYNERGISTIC ACTION OF TWO OXYSTEROLS IN THE LOWERING OF HMG-CoA REDUCTASE ACTIVITY IN CHO-K1 CELLS*

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Summary: 3β-Hydroxy-5α-cholest-8(14)-en-15-one (**I**) and (25R)-26-hydroxycholesterol (**II**), both potent regulators of sterol biosynthesis, have been found to show synergism in the reduction of the levels of HMG-CoA reductase activity in CHO-K1 cells. When equimolar concentrations of **I** and **II** were added in combination, synergistic reduction ($p<0.0001$) of enzyme activity was observed at total oxysterol concentrations of 0.1 μM, 0.2 μM, and 0.5 μM. Maximal synergistic effect in the lowering of reductase activity (28% greater than predicted) was observed at 0.1 μM total oxysterol concentration. Five additional experiments conducted with 50 nM oxysterols confirmed the synergistic effect at 0.1 μM total sterol concentration. These results suggest that the *in vivo* importance of **I** and **II** may be greater than that anticipated on the basis of the concentrations of the individual sterols.

3β-Hydroxy-5α-cholest-8(14)-en-15-one (**I**) (1-4) and (25R)-26-hydroxycholesterol (**II**) (5,6) are potent regulators of sterol synthesis and reduce the levels of HMG-CoA reductase activity in cultured mammalian cells. Both sterols show very high affinity binding to an oxysterol binding protein believed to be important in the regulation of HMG-CoA reductase activity (6). In addition, the natural occurrence in mammals of both **I** (7) and **II** (8-11) has been reported. Thus, both **I** and **II** represent potential natural regulators of sterol and isoprenoid biosynthesis, and consequently represent potential regulators of cell replication (12,13). In this report, we present evidence that indicates that **I** and **II** act synergistically to lower the levels of HMG-CoA reductase activity in CHO-K1 cells.

MATERIALS AND METHODS

Thin layer chromatography (TLC) was carried out on plates of silica gel G (Analtech, Newark, DE) or Whatman LK5D silica gel (American Scientific Products, Houston, TX). Components on the plates were detected after spraying with molybdic acid (14). **I**, prepared by a modification (15) of a procedure described previously (1,16), showed a single component on TLC (solvent systems: benzene and hexane-ethyl acetate, 3:7). **II**, a generous gift from Syntex, Inc. (Palo Alto, CA), showed a single component on TLC in 5 solvent systems (hexane-ethyl acetate

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Abbreviation: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

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(9:1), chloroform, methanol-ethyl acetate (1:1), ethyl acetate, and ethyl acetate-methanol-acetic acid (7:3:1)). (3RS)-[3-^{14}C]HMG-CoA (56 mCi per mmol) and (3RS)-[2-^{3}H]mevalonolactone (176 mCi per mmol) were purchased from the Amersham Corporation (Arlington Heights, IL). All reagents for the assay of HMG-CoA reductase were obtained from Sigma Chemical Company (St. Louis, MO). Trypsin was purchased from Gibco Laboratories (Grand Island, NY) and Lux tissue culture plastic was obtained from Miles Scientific (Elkhart, IN). Powdered Ham's F12 medium (17) and phosphate-buffered saline (PBS; KCl 2.7 mM; KH_2PO4 1.2 mM; NaCl, 137 mM, and $NaHPO_4$, 8.1 mM) were purchased from Irvine Scientific (Irvine, CA). Fetal calf serum was obtained from Whittaker M.A. Bioproducts (Elkhart, IN). Sterols were dissolved in ethanol (10 mg per ml) and appropriate aliquots of stock solutions were added to Ham's F12 medium supplemented with 5% delipidated (18) fetal calf serum (lipid-deficient medium) and allowed to equilibrate for at least 6 h at room temperature prior to storage at 4° C. Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Ham's F12 medium supplemented with 5% fetal calf serum (lipid-rich medium) in a humidified atmosphere of 5% CO_2-95% air at 37° C. Each experiment was initiated by inoculating 3.75 x 10^5 cells into 100 mm dishes containing lipid-rich medium (10 mls) followed by incubation for 48 h. The medium was aspirated and, after rinsing of the plates with PBS (10 ml), lipid-deficient medium (10 ml) was added to each plate, and the cells returned to the incubator for 18 h. The lipid-deficient medium was removed and fresh lipid-deficient medium containing sterols (or the appropriate quantities of ethanol in the case of controls) was added to the plates and the incubation continued for 4 h. Cells were harvested by scraping, and detergent-solubilized cell preparations were obtained for the assay of HMG-CoA reductase activity using the method of Brown *et al.* (19). Assays were carried out as described by Pinkerton *et al.* (3) except that the specific activity of (3RS)-[3-^{14}C]HMG-CoA was 20,000 dpm per nmol. Protein was determined by the method of Lowry *et al.* (20) after precipitation with trichloroacetic acid.

All statistical analyses were performed on Macintosh computers using the Statview statistical program. The enzyme activities obtained from oxysterol-treated cells were normalized to enzyme activities obtained from control cells (i.e. cells untreated with oxysterols) according to the equation:

$$\text{(Eq. 1)} \quad \% \text{ Inhibition} = \left(1 - \frac{\text{enzyme activity}_E}{\text{enzyme activity}_C}\right) \times 100,$$

where E is the enzyme activity at a given concentration of experimental oxysterol added and C is the value obtained in the absence of exogeneously added oxysterol. The data base was also normalized to both control values and to maximally inhibited values (double normalization) according to the equation:

$$\text{(Eq. 2)} \quad \text{Activity} = \frac{\text{enzyme activity}_E - \text{enzyme activity}_M}{\text{enzyme activity}_C - \text{enzyme activity}_M},$$

where E is a sterol concentration, C is the appropriate control value, and M is the value obtained at the oxysterol concentration causing maximal inhibition. The value of the doubly-normalized percent suppression of enzyme activity by a given oxysterol concentration is then given by the equation:

$$\text{(Eq. 3)} \quad \% \text{ Suppression} = (1 - \text{Activity}) \times 100.$$

These operations were performed separately for each replicate enzyme assay using the basal control (no added oxysterol) and maximally inhibited values for that experiment. Concentration-response curves revealed that in all cases maximally inhibited values were in a plateau region. The percent inhibition of enzyme activity calculated for each concentration of sterol added separately was used to calculate the predicted inhibition of enzyme activity by the two sterols combined. This calculation was based on the assumptions that the separate effects of the oxysterols were additive, and that there was a linear relationship between the concentrations of each sterol and the doubly normalized values of the enzyme activity. The latter assumption was tested by linear regression analysis, and R values of 0.75 and 0.73 were obtained for **I** and **II**, respectively.

RESULTS

CHO-K1 cells, incubated for 18 h in lipid-deficient medium to elevate HMG-CoA reductase activity, were then incubated for 4 h with **I**, **II**, or both sterols combined, and the levels of HMG-CoA reductase activity were determined. The results of 4 separate experiments indicated that both **I** and **II**, when added separately, reduced the levels of HMG-CoA reductase activity in a

concentration-dependent fashion. Statistical analyses (Analysis of Variance, ANOVA) showed a highly significant correlation ($p<0.0001$) between sterol concentration and reduction of enzyme activity over the range of 0.1 to 0.5 μM.

In order to determine whether **I** and **II** in combination exerted a synergistically inhibitory effect on HMG-CoA reductase activity, it was necessary to calculate the predicted inhibition caused by the combined sterols at each concentration. This calculation was based on the assumption that for each sterol, the goodness of fit of the relationship between the reduction of enzyme activity and the oxysterol dose was sufficient so that the inhibitory effects of the sterols could be calculated in a simple additive fashion. Values were calculated for the predicted suppression of HMG-CoA reductase by **I** and **II** combined for each concentration tested. Since a model of simple additivity was postulated, the commutative operation was performed: multiplying the observed, individual degrees of inhibitory activity using doubly-normalized enzyme activities. The percent suppression was then calculated using Eq. 3.

The results of four experiments are presented in Figure 1 and show that, at total concentrations of 0.1, 0.2, and 0.5 μM of the sterols in the culture medium, the extents of suppression of HMG-CoA reductase activity were greater than the predicted values, i.e., the oxysterols were reducing enzyme activity in a synergistic fashion. At 0.1 μM total sterol concentration (0.05 μM each sterol), the observed suppression was 28% greater than the predicted value ($p<0.0001$; two factor ANOVA). At 0.2 μM total sterol concentration, the observed suppression was 19% greater than the predicted value ($p<0.0001$; two factor ANOVA) and at 0.5 μM total sterol concentration, it was 7% greater than the predicted value ($p<0.0001$; two factor ANOVA).

When equimolar concentrations of **I** and **II** were incubated with the cells, maximum suppression of enzyme activity occurred at 0.5 μM total sterol concentration, i.e., 0.25 μM **I** plus 0.25 μM **II**. Addition of higher concentrations of the oxysterols (up to 1.00 μM of each sterol) did not lead to significantly greater reduction of enzyme activity. Analysis of variance (three factor

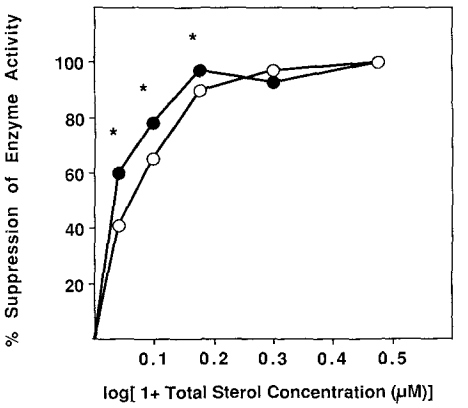

Figure 1. Synergistic effects of I and II in lowering HMG-CoA reductase activity in CHO-K1 cells incubated in lipid-deficient medium. Cells were grown and reductase assays were performed as described in the text. Data points were calculated from doubly normalized data as described in the text. Significant differences (*) between the observed values (●-●) and the predicted values (O-O) were obtained at total oxysterol concentrations of 0.1 μM (28%), 0.2 μM (19%), and 0.5 μM (7%) in the medium.

ANOVA) showed that the suppression of HMG-CoA reductase activity caused by the combination of the two sterols was greater than that caused by either **I** alone ($p<0.0001$) or **II** alone ($p<0.0001$) over the concentration 0.1 μM to 0.5 μM total oxysterol in the medium (Figure 1).

Five additional experiments were carried out at 50 nM concentrations of **I** and **II**, added separately and in combination at a total oxysterol concentration of 50 nM. Statistical analyses of the doubly-normalized data were performed as described above. Significant synergistic ($p=0.005$) reduction of HMG-CoA reductase activity was again observed when 50 nM each of **I** and **II** were added to the culture medium.

DISCUSSION

These studies demonstrate that **I** and **II** exerted a synergistic effect in the lowering of HMG-CoA reductase activity in Chinese hamster ovary cells. To our knowledge, such a synergistic action of two oxygenated sterols on HMG-CoA reductase activity has not been reported previously.

The mechanism(s) involved in this synergistic action is not known. Both **I** (1-4) and **II** (5,6) are very potent regulators of the levels of HMG-CoA reductase in cultured mammalian cells. In contrast to the cases of competitive inhibitors of HMG-CoA reductase, the direct addition of **I** to enzyme preparations had no detectable effect on HMG-CoA reductase activity (21). Both **I**, **II** and 25-hydroxycholesterol (**III**) have been shown to bind very tightly to a cytosolic oxysterol binding protein which has been reported to be involved in the suppressive effects of a number of oxygenated sterols (including **I**, **II**, and **III**) on the levels of HMG-CoA reductase activity in cultured cells (6). Two mutants resistant to the effects of **III** were also resistant to the effects of **I** on HMG-CoA reductase activity (3,22). These findings suggest that **I**, **II**, and **III** share at least some common features in the suppression of enzyme activity.

The mechanisms involved in the action of oxysterols on HMG-CoA reductase activity in cultured mammalian cells appear to be complex, involving processes controlling both the synthesis and the degradation of the enzyme (23-26). The expression of these processes is under nuclear control (27). It should also be noted that the magnitude of the inhibitory action of an individual oxygenated sterol is the composite of a number of factors including, but not necessarily limited to, the rate of uptake of the sterol by the cells, the nature of the interaction of the sterol with the oxysterol-binding protein(s), rates of possible metabolism of the oxygenated sterol to species of higher, lower, or unchanged activity with respect to effects on HMG-CoA reductase activity, and additional sites of action (other than the suppression of HMG-CoA reductase) which might lead to a secondary modulation of reductase activity.

There are a number of potential points which could lead to the synergistic action of **I** and **II** on reductase activity. One of the sterols may affect the uptake and/or metabolism of the other, and allosteric effects on the oxysterol binding protein(s) cannot be excluded. Alternatively, the synergy of **I** and **II** may be the consequence of the interaction of discrete pre- and post translational effects of the two sterols. Further studies on the molecular and cellular effects of these regulatory sterols will be required to define the mechanism(s) and importance of the synergistic action of **I** and **II**. However, the demonstration that two highly active natural regulators of cholesterol biosynthesis

interactively reduce the level of activity of a key regulatory enzyme in sterol biosynthesis implies that their *in vivo* significance may be greater than that anticipated on the basis of the concentrations of the individual sterols. Moreover, since oxysterols in blood (11) and in cells (28,29) exist as mixtures, our results indicate the importance of consideration of possible interactive effects of the oxysterols in the expression of their actions in cells and in intact animals.

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