Vol. 120, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1984 Pages 192-198

# GROWTH-RATE-RELATED AND HYDROXYSTEROL-INDUCED CHANGES IN MEMBRANE FLUIDITY OF CULTURED HEPATOMA CELLS : CORRELATION WITH 3-HYDROXY-3-METHYL GLUTARYL CoA REDUCTASE ACTIVITY

Lysiane RICHERT*, Monique CASTAGNA***, Jean-Paul BECK*, Shihong RONG**, Bang LUU** and Guy OURISSON**

*Institut de Physiologie et de Chimie Biologique,
**Centre de Neurochimie du CNRS,
Université Louis Pasteur, 67084 STRASBOURG - FRANCE
***Institut de Recherches Scientifiques sur le Cancer,
94800 VILLEJUIF - FRANCE

Received February 16, 1984

SUMMARY : 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.3.4.) activity and cell membrane fluidity measured by fluorescence polarization using 1,6 diphenyl, 1,3,5-hexatriene as fluorescent probe have been concomitantly examined in HTC hepatoma cells, both in relation to growth rate and in response to treatment with hydroxylated sterols. A high level of HMG-CoA reductase activity was observed in cells at log phase of growth which progressively decreased to reach a sustained low level at stationary phase. Similarly, membrane fluidity markedly decreased in relation to growth rate. Hydroxylated sterols such as 7 ß-hydroxycholesterol or 25-hydroxycholesterol strongly inhibited HMG-CoA reductase activity whereas a water-soluble derivative of 7 ß-hydroxycholesterol sodium 3,7-bishemisuccinate had no effect. Within the same range of concentrations 7 ß-hydroxycholesterol and 25-hydroxycholesterol strongly decreased membrane fluidity when the water-soluble derivative was ineffective. Thus, the present results provide evidence for a correlation between the two tested parameters and suggest a dependency of HMG-CoA reductase activity on cell membrane fluidity.

Membrane fluidity is currently considered as being a crucial regulator of membrane functional dynamics : some cellular processes such as proliferation (1,2), fusion (3) and endocytosis (4) are associated with changes in membrane fluidity. Studies involving a broad range of membrane-bound enzymes have shown that a decreased membrane fluidity in cholesterol-enriched membranes resulted in a strong inhibition of enzyme activity (5,7) whereas an increased membrane fluidity, as a result of various manipulations, had a reverse effect (8).

Hydroxylated sterols are known as potent inhibitors of sterol synthesis in a variety of mammalian cells (9-14). Their mechanism of action is not precisely known. These compounds suppress the activity of HMG-CoA

0006-291X/84 $1.50

reductase*, the rate limiting enzyme of the sterol synthetic pathway, which catalyses the conversion of HMG-CoA to mevalonate. However, it has been shown that 25-hydroxycholesterol, one of the most active inhibitors of sterol synthesis, does not affect directly the enzyme in an **in vitro** system (15-17) but requires intact cells for being active. It has also been established that hydroxylated sterols specifically bind to a cytosolic protein (18-20) without suppressing synthesis nor enhancing degradation of the enzyme (21) located in the endoplasmic reticulum (22). It has been recently reported that hydroxylated sterols associate with microsomal membranes, thus suggesting that the inhibitory effect of these sterols on HMG-CoA reductase could be mediated through alterations of membrane dynamic properties (23).

The present study was undertaken in order to concomitantly investigate the effect of two lipophilic hydroxylated sterols, 25-HC and 7-HC on both HMG-CoA reductase and cell membrane fluidity in cultured HTC cells. For the purpose of comparison, a newly synthetized hydrophilic derivative, 7-HC BHS, was similarly tested. We found that hydroxylated sterols such as 25-HC and 7-HC strongly decreased membrane fluidity and inhibited the enzyme whereas 7-HC BHS did not impair membrane fluididy or enzyme activity. In addition we report results which provide evidence for similar correlative changes between cell membrane fluidity and HMG-CoA reductase activity in relation to growth rate of HTC cells.

## MATERIALS AND METHODS

HTC cells were grown in 75 ml culture flasks with magnetic stirring as previously described (24) in Swim's 77 medium containing 10% newborn calf serum(Gibco). Cells were numbered using a Neubauer microcytometer. The hydroxylated sterol 7-HC and the hydrophilic derivative 7-HC BHS were synthesized in our laboratory (25) and 25-HC was kindly suplied by Hoffmann-La Roche. Sterols were dissolved either in ethanol (7-HC and 25-HC) or in water (7-HC BHS) and added to the cultures in 100 µl solution at various concentrations. The addition of 100 µl ethanol to control cultures did not affect either enzyme activity or fluorescence polarization. The fluorescence measurements were conducted essentially as previously described (26) and slightly modified (27). Cells ($10^6$) were labelled with DPH (2µM) dispersed in 1.5 ml phosphate-buffered solution by incubation for 30 min at 25°C. The fluorescence polarization of the samples was analyzed with a microviscosimeter Elscint, model MV-1, which directly recorded the degree of fluorescence polarization P.

HMG-CoA reductase activity was assayed according to Kandutsch **et al** (10) including the following modifications : cells (5 X $10^6$) were first disrupted by sonication in 50mM phosphate buffer at pH 7.4 containing 5 mM DDT, 0.2 M KCl and 0.25 % Kyro EOB detergent. The final reaction mixture

*The **abbreviations used are** : HMG-CoA : 3-hydroxy-3-methylglutaryl-coenzyme A ; DPH : 1,6, diphenyl 1,3,5, hexatriene ; 25-HC : 25-hydroxycholesterol ; 7-HC : 7 ß-hydroxycholesterol ; 7-HC BHS - sodium 3,7-bishemisuccinate of 7 ß-hydroxycholesterol.

contained 20 µl cell homogenate, 5mM DTT, 10mM glucose-6-phosphate dehydrogenase in a 0.2 ml volume. The reaction was initiated by the addition of 25 µl of DL-3-[glutaryl-3-$^{14}$C]-hydroxy-3-methylglutaryl CoA (New England Nuclear, spec. act. 58,6 Ci/M, 10 µCi/ml) and proceeded for one hour at 37°C. The reaction was stopped by 25 µl 5N HCl. Then 25 µl of R-[5-$^{3}$H] mevalonate (New England Nuclear, spec. act. 4 Ci/mM, 10 µCi/ml) was added and lactonization was allowed to proceed at 20°C for 15 hours. Mevalonolactone was separated from mevalonic acid and HMG-CoA by chromatography on silica gel 60F-25H TLC plates (Merck) with toluene/chloroform 1:1) as solvent mixture. The spot of mevalonolactone was scrapped from the chromatography plate and collected in 5 ml scintillation fluid. $^{14}$C and $^{3}$H radioactivities were analyzed in a Beckman scintillation counter and the amount of $^{14}$C mevalonate formed was calculated as already described (28). Protein content was measured according to Lowry's technique and HMG-CoA reductase activity was expressed as nmol of mevalonate formed per hour per mg cell protein.

## RESULTS

### 1) Membrane fluidity and HMG-CoA reductase activity in HTC cells at different growth phase.

HTC Cells grew exponentially during the two first days of culture when the cellular concentration was initially adjusted to $10^5$ cells per ml culture medium. Then growth rate slowed down to the saturating density of approximately $10^6$ cells per ml which was reached after 4 to 5 days of culture. In fast growing cells a high level of HMG-CoA reductase activity was detected (6.4 nm/h/mg of protein) which progressively decreased in relation to time and reached the lower level (1.2 nml/h/mg of protein) at day 4, that means at stationary growth phase (Fig. 1). Parallel studies of fluorescence polarization have indicated that cell membranes were more fluid in fast growing phase (P = 0.210) than in stationary growth phase (P = 0.240), as shown in Table 1.

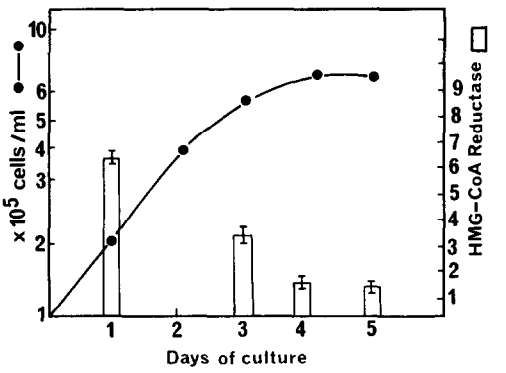


**Figure 1** : Growth curve of HTC cells in suspension culture (●——●) and HMG-CoA reductase activity expressed as nmol of mevalonate formed/ h/mg cell protein ( □ ). The results represent the mean values from 3 different experiments.

TABLE 1 : DEGREE OF FLUORESCENCE POLARIZATION IN HTC CELLS AT DIFFERENT GROWTH PHASES

| Time of culture | day 1 | day 4 | day 5 |
|---|---|---|---|
| fluorescence polarization (P) | 0.210 ± 0.003 | 0.240 ± 0.005 | 0.241 ± 0.004 |

Fluorescence polarization measurements were determined as described in Materials and Methods. Cells collected at day 1 were exponentially growing whereas cells collected at days 4 and 5 reached the stationary growth phase.

### 2) Membrane fluidity and HMG-CoA reductase activity in hydroxysterol treated cells

The two tested sterols 25-HC and 7-HC evoked a higher degree of fluoresence polarization in treated cells as compared with the control cells, indicating a lower membrane fluidity. The most active compound, 25-HC, increased P from 0.210 to 0.240 at a concentration of 40 µM whereas 7-HC was uneffective below 80 µM. Under the same conditions the hydrosoluble derivative 7-HC BHS had no effect on membrane fluidity (Fig. 2a). The higher degree of fluorescence polarization was reached 90 min after the addition of

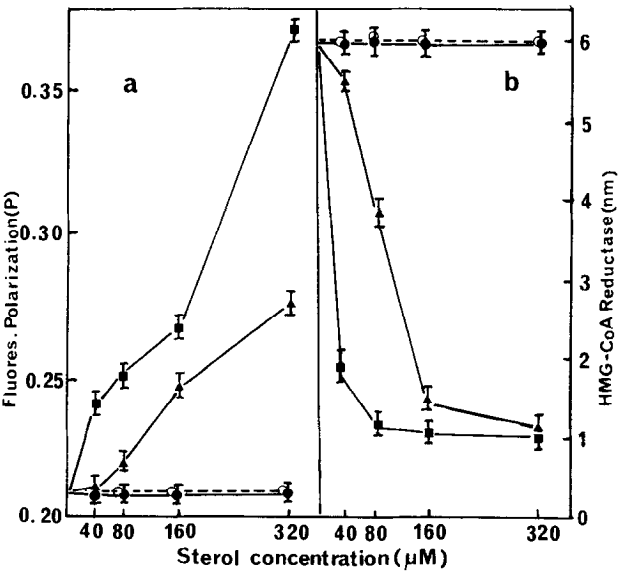


Figure 2 : Effect of various hydroxylated sterols upon fluorescence polarization and HMG-CoA reductase activity. The degree (P) of fluoresence polarization (Fig. 2a) and the enzyme activity (Fig. 2b) were measured as described in Materials and Methods. Cells were treated after one day of culture for 90 min with various concentrations of 25-HC (■——■), 7-HC (▼——▼) and 7-HC BHS (●——●) and compared with untreated cells (○- -○). The results represent the mean values from 3 different experiments.

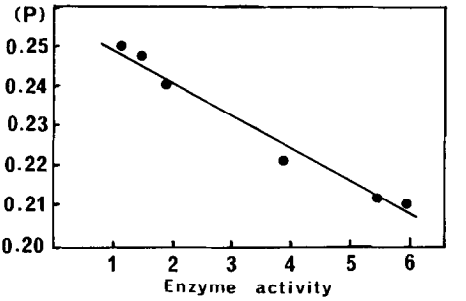


**Figure 3** : Correlative changes between HMG-CoA reductase activity and the degree of fluorescence polarization in hydroxysterol-treated cells at concentrations within the range of dose dependency. Enzyme activities and P values were drawn from Fig. 2a and b.

either 25-HC or 7-HC at the concentration of 160 μM and plateaued for at least 5 h (data not shown). Within the same range of concentrations, the treatment with hydroxylated sterols strongly inhibited HMG-CoA reductase activity. At the concentration of 80 μM, 25-HC maximally inhibited the enzyme activity which dropped from about 6 to 1 nmol/h/mg cell protein. Again 7-HC was less active and only inhibited the enzyme activity maximally at the concentration of 160 μM. In contrast, 7-HC BHS did not inhibit the enzyme activity at any used concentrations (Fig. 2b). The inhibitory effect of hydroxysterols on HMG-CoA reductase activity plateaued at the concentration of 160 μM for 7-HC and 80 μM for 25-HC. Within the range of dose-dependent inhibition of HMG-CoA reductase activity the plot of enzyme activity versus cell membrane fluidity actually showed a rather good correlation between the two parameters (Fig. 3).

## CONCLUSION

The present study has shown that spontaneous variations of HMG-CoA reductase activity occur in HTC cells in relation to growth rate which are correlated with changes in cell membrane fluidity. Such a relationship between membrane dynamic properties and HMG-CoA reductase activity was similarly assessed using various hydroxysterols which differently influence the enzyme activity. 7-HC and 25-HC, both increased the degree of fluorescence polarization indicating a lower membrane fluidity and as well inhibited the enzyme activity. Under similar conditions, the water-soluble derivative 7-HC BHS was uneffective on either parameters.

The biosynthesis of cholesterol seems to be regulated in liver cells by many ways all of which affect HMG-CoA reductase, the enzyme catalyzing the rate limiting step (29). Cholesterol appears to regulate this pathway since dietary cholesterol inhibits cholesterol biosynthesis (30). Moreover recent studies have shown that cholesterol decreased HMG-CoA

reductase activity in isolated microsomes and enhanced acyl-CoA-cholesteryl transferase, a microsomal enzyme responsible for sterol esterification ((31, 32). The authors have proposed that cholesterol-induced modulation is probably related to an increased rigidity of liquid domains of the membrane. Hydroxysterols affect many cellular processes, the mechanisms of which are still unclear. Some of them like the formation of E. rosettes in lymphocytes (33) or the conversion of red cells to echinocytes (34) suggested that these sterols cause specific changes at the membrane level. Hydrosterols are esterified at a similar rate as cholesterol (23). Therefore it is suggested that these compounds may insert into the membrane and replace cholesterol in its modulating function of HMG-CoA reductase activity.

A recent report from our laboratory has shown that lipophilic 7-HC and its hydrophilic derivative 7-HC BHS inhibited rapidly DNA synthesis under the conditions used in the present experiments (35). Thus, the finding favors the possibility that membrane fluidity and HMG-CoA reductase activity do not directly correlate with DNA synthesis. The mechanism of action of the newly synthetized 7-HC BHS is quite unknown. The present results suggest that this water-soluble compound, unlike 7-HC and 25-HC does not insert into membranes, specifically smooth microsomal membranes where HMG-CoA reductase has been located (32).

**ACKNOWLEDGEMENTS** : We are grateful to Mrs. M.T. BOHN for her technical assistance. This research was supported by grants from the "Fondation pour la Recherche Médicale", from the "Fédération Nationale des Centres de Lutte contre le Cancer" and from the INSERM-CRE n°832002.

## REFERENCES

1. Inbar, M., Yuli, I., and Raz, A. (1977) Exptl. Cell. Res. **105**, 325-335.
2. Muller, C.P., Volloch, Z., and Schinitzky, M. (1980) Cell. Biophys. **2**, 233-240
3. Prives, J., and Shinitzky, M. (1977) Nature **268**, 761-763.
4. Heiniger, H.J., Kandutsch, A.A., and Chen, H.W. (1976) Nature **263**, 515-517.
5. Graham, J.M., and Green, C. (1970) Eur. J. Biochem. **12**, 58-66.
6. Kimelberg, H.K. (1975) Biochim. Biophys. Acta **413**, 143-156.
7. Farias, R.N., Bloj, B., Morero, R.D., Sineriz, F., and Trucco, R.E. (1975) Biochim. Biophys. Acta **415**, 231-251.
8. Houslay, M.D., Dipple, I., and Gordon, L.M. (1981) Biochem. J. **197**, 675-680.
9. Kandutsch, A.A., and Chen, H.W. (1977) J. Biol. Chem. **252**, 409-415.
10. Breslon, J.L., Lothor, D.A., Spauling, D.R., and Kandutsch, A.A. (1975) Biochim. Biophys. Acta **398**, 10-17.
11. Astruc, M., Laporte, M., Tabacik, C., and Crastes de Paulet, A. (1978) Biochem. Biophys. Res. Comm. **85**, 691-700.
12. Kandutsch, A.A., Chen, H.W., and Heiniger, H.J. (1978) Science **201**, 498-501.
13. Yachnin, S., Streuli, R.A., Gordon, L.I., and Ksu, R.C. (1979) Current Topics of Hematology **2**, 245-271.
14. Zander, M., Koch, P., Luu, B., Ourisson, G., and Beck, J.P. (1977) J. Chem. Res. (M), 2572-2583.
15. Kandutsch, A.A., and Chen, H.W. (1974) J. Biol. Chem. **249**, 6057-6061.

16. Kandutsch, A.A., and Chen, H.W. (1973) J. Biol. Chem. **248**, 8408-8417.
17. Sinensky, M., Duwe, G., and Pinkerton, F. (1979) J. Biol. Chem. **254**, 4482-4486.
18. Kandutsch, A.A., Chen, H.W., and Shawn, E.P. (1977) Proc. Natl. Acad. Sci. USA **74**, 2500-2503.
19. Kandutsch, A.A., and Thompson, E.B. (1980) J. Biol. Chem. **255**, 10803-10826.
20. Sinensky, M., and Mueller, G. (1981) Arch. of Biochem. and Biophys. **209**, 314-320.
21. Saucier, S.E., and Kandutsch, A.A. (1979) Biochim. Biophys. Acta **572**, 541-556.
22. Bucher, L.R., Overath, P., and Lynew, F. (1960) Biochim. Biophys. Acta **40**, 491-501.
23. Lichtenstein, A., and Brecher, P. (1983) Biochim. Biophys. Acta **751**, 340-348.
24. Thompson, E.B., Tomkins, G.M., and Curran, J.F. (1966) Proc. Natl. Acad. Sci. USA **56**, 296-303.
25. Rong, S. (1983) Doctoral dissertation, University Louis Pasteur Strasbourg
26. Inbar, M., and Shinitzky, M. (1974) Proc. Natl. Acad. Sci. USA **71**, 4229-4231.
27. Castagna, M., Rochette-Egly, C., Rosenfeld, C., and Mishal, Z. (1979) FEBS Letters **100**, 62-66.
28. Goldfarb, S., and Pitot, H.C. (1971) J. Lipid. Res. **12**, 512-515.
29. Rodwell, V.W., Norstrom, J.L., and Mitschelen, J.J. (1976) Adv. Lipid. Res. **14**, 1-74.
30. Gould, R.G., Taylor, C.B., Hagerman, J.S., Warner, I., and Campbell, D.G. (1953) J. Biol. Chem. **201**, 519-528.
31. Mitropoulos, K.A., Venkatesan, S., Reeves, B.E.A., and Balasubramaniam, S. (1981) Biochem. J. **194**, 265-271.
32. Hasmimoto, S., Drevon, C.A., Weinstein, D.B., Bernett, J.S., Dayton, S., and Steinberg, D. (1983) Biochim. Biophys. Acta **754**, 126-133.
33. Streuli, R.A., Chung, J., Scanu, A.M., and Yachnin, S. (1979) J. Immunol. **123**, 2897-2902.
34. Hsu, R.C., Kanofsky, J.R., and Yachnin, S. (1980) Blood **56**, 109-115.
35. Richert, L., Bergmann, C., Beck, J.P., Rong, S., Luu, B., and Ourisson, G. (1983) Biochem. Biophys. Res. Comm. **117**, 851-859.