Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 335-341

REGULATION OF SQUALENE SYNTHETASE ACTIVITY IN RAT LIVER: ELEVATION BY CHOLESTYRAMINE, BUT NO DIURNAL VARIATION

L.H. Cohen*, A.M. Griffioen*, R.J.A. Wanders**, C.W.T. Van Roermund**, C.M.G. Huysmans*, and H.M.G. Princen*

*TNO Gaubius Institute for Cardiovascular Research, Herenstraat 5d, 2313 AD Leiden, The Netherlands

**Department of Pediatrics, University Hospital Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

Received May 30, 1986

Squalene synthetase activity in liver microsomes from rats sacrificed at three different times of the diurnal cycle showed no significant differences. Addition of 4% cholestyramine to the food resulted in a marked increase in activity (280% of control), independent of the time of killing. 3-Hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol 7α-hydroxylase activity, determined as positive controls, were also found to be elevated by cholestyramine and additionally showed a diurnal variation. On the other hand, five control enzyme activities, not directly related to cholesterol metabolism, i.e. glutamate dehydrogenase, NADPH cytochrome-c reductase, β-hexosaminidase, catalase and acyl coenzyme A oxidase, showed neither an influence of cholestyramine feeding nor a time of sacrifice dependent variation.

Besides 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase (EC 1.1.1.34), which is the major rate limiting step in cholesterol biosynthesis (1), squalene synthetase (EC 2.5.1.21) has been suggested to be a secondary regulation site in this pathway. This was based on the observations, that long-term cholesterol feeding reduced the conversion of farnesyl pyrophosphate to cholesterol but not that of squalene to cholesterol (2) and that fasting and refeeding affected squalene synthetase activity (3) as determined in rat liver. The enzyme activity was also reported to be suppressed by low density lipoprotein in human fibroblasts (4), leading to the supposition that the regulation of squalene synthetase could play a role in maintaining sufficient substrate levels for the biosynthesis of non-sterol side products (5).

The bile acid sequestrant cholestyramine stimulates the cholesterol synthesis rate in rat liver (6,7), due to a strong increase of the HMGCoA

0006-291X/86 $1.50

reductase activity. Because of the diurnal variation in the activity of this enzyme, the stimulation is maximal in the middle of the dark period (8). We decided to study the activity of squalene synthetase in rat liver microsomes during the diurnal cycle in response to cholestyramine feeding.

Materials and methods. All radiochemicals were obtained from New England Nuclear. Bristol Myers supplied Questran (cholestyramine). All other chemicals were of the highest purity available.

Animals: Male Wistar rats, weighing 200-300 g, were kept on either a normal (6 a.m. - 6 p.m. light, 6 p.m. - 6 a.m. dark) or a reversed (6 a.m. - 6 p.m. dark, 6 p.m. - 6 a.m. light) light regime and received conventional rat pellets ad libitum during two weeks. During the third week the rats received either a normal diet or the same supplemented with 4% (w/w) cholestyramine. Rats were killed at 10 a.m. (both light regimes) and at 3 p.m. (rats on the normal light regime only; n = 4 for groups of identically treated rats).

Preparation of rat liver homogenates and microsomes: Rats were anesthetized with Nembutal, followed by liver perfusion with cold 0.1 M sucrose - 0.05 M KCl - 30 mM EDTA - 5 mM DTT - 0.04 M potassium phosphate (pH = 7.4). Microsomes (washed 100.000 x g pellets) were prepared in the presence of either 50 mM KCl or 50 mM KF (a phosphatase inhibitor) according to Edwards and Gould (9). A sample of the liver homogenates in perfusion buffer was frozen in N_2(l) and stored at -80°C. The microsomal preparations, used for the assay of squalene synthetase activity, were resuspended in 30 mM nicotinamide - 5 mM $MgCl_2$ - 10 mM KF - 0.1 M potassium phosphate (pH = 7.4), frozen in aliquots in N_2 (l) and stored at -80°C for several months without loss of activity.

Preparation of Farnesyl-[3,7,11-^{14}C]-pyrophosphate: [^{14}C]-Farnesylpyrophosphate was prepared enzymically from R-[3-^{14}C]-mevalonolactone essentially as described by Popjàk et al. (10). Pure farnesylpyrophosphate (a gift of Dr. C.D. Poulter) was used as a reference. The specific radioactivity was 1767--2705 dpm/nmol and the radiochemical purity was 97-98% as determined by HPLC chromatography.

Assay of squalene synthetase activity: Incubations were carried out essentially according to Popjàk (9) with the following modifications. After preincubation of 90 µl of the incubation mixture for 3 min at 37°C the reaction was started by addition of 10 µl of the substrate solution. See under Results for incubation conditions. All solutions were equilibrated with nitrogen gas and the incubations performed under nitrogen. The incubations were stopped by the addition of 150 µl 5 M NaOH. [^{3}H]Squalene (80.000 dpm) as a recovery standard and unlabelled squalene (5 µg) as carrier were added and the lipids were extracted according to Bligh and Dyer (12) in the presence of 0.005% w/v butylated hydroxytoluene as an antioxidant. The lipids were separated by TLC on silicagel-60 plates (0.25 mm). The chromatograms were developed for 2.5 cm in chloroform:methanol:water = 75:25:4, dried under nitrogen and subsequently developed in hexaan:ether = 49:1 (13). The area containing squalene was scraped into scintillation vials and the ^{3}H/^{14}C-radioactivity was determined using instafluor. The ^{14}C-cpm of the squalene formed was corrected for the recovery of [^{3}H]-squalene, which was in between 50 and 70%. Enzyme activity is expressed in nmol of squalene formed/min per mg of microsomal protein. The values given are the averages of duplicate determinations, which agreed within 10%.

Other enzyme activity assays: HMGCoA reductase activity was determined essentially according to Philipp and Shapiro (14). Duplicate 30 min incubations in the presence and absence of cofactors were performed. Values obtained in the presence of cofactors were corrected for the counts formed in the absence of cofactors. Cholesterol 7α-hydroxylase (EC 1.14.13.7) activity was measured as described by Goodwin et al. (15). AcylCoA oxidase (EC 6.2.1.3) activity was assayed according to (16), except that the reaction mixture

contained: 50 mM triethanolamine-HCl, pH = 7.8, 15 mM phenol, 1 mM amino-antipyrine, 10 µM FAD, 0.5 mM NaN_3, 0.01% (w/v) Triton X-100, 10 IU/ml horse-radish peroxidase, 0.15 mg/ml BSA and 0.1 mM coenzyme A. NADPH-cytochrome c reductase (EC 1.6.2.3) activity was determined according to Beaufay et al. (17) with additional 0.1% (w/v) Triton X-100 and 1 µM rotenon in the reaction mixture. Catalase (EC 1.11.1.6) activity and β-hexosaminidase (EC 3.2.1.52) activity were measured as described previously (18). Glutamate dehydroxygenase (EC 1.4.1.2) activity was assayed by measuring the decrease in extinction at 340 nm in a mixture containing: 50 mM triethanolamine, 100 mM NH_4Cl, 1 mM ADP, 0.3 mM NADH, 2.5 mM EDTA, 0.1% Triton X-100. The reaction was started by the addition of 10 mM ketoglutamate at pH = 8.0. Protein concentrations were determined as described by Lowry et al. (19).

Results and discussion

Squalene synthetase assay in rat liver microsomal preparations: The determination of squalene synthetase activity in rat liver microsomes was made more accurate by the use of [^{3}H]-squalene as a recovery standard (see under Materials and Methods). As is depicted in Figure 1a the enzyme in microsomal preparations, obtained from cholestyramine treated rats, appears to be saturated at substrate concentrations higher than 0.04 mM. In contrast with the enzymes from pig liver (20) and yeast (21), the rat liver enzyme showed only a very moderate substrate inhibition at farnesylpyrophosphate concentrations above 0.1 mM. At a substrate concentration of 0.07 mM squalene formation was linear with incubation time up to 30 min (Fig. 1b). For microsomal protein concentrations up to 1 mg/ml linearity for enzyme activity with

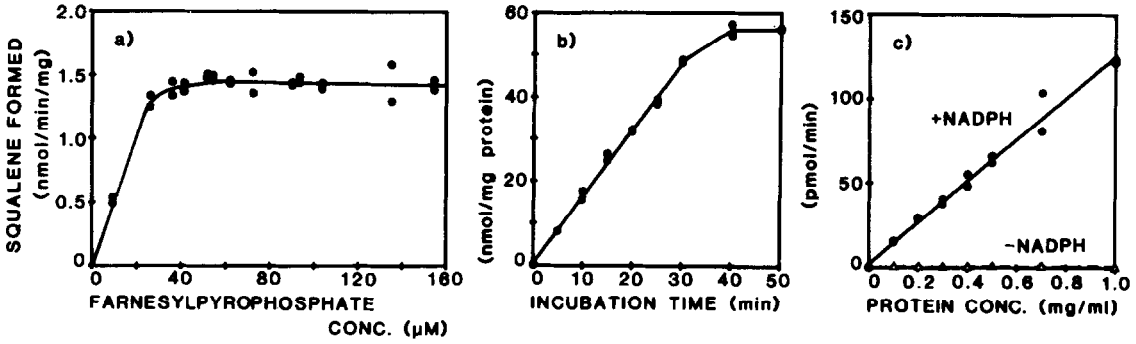

Figure 1: [^{14}C]-Squalene synthesis from [^{14}C]-farnesylpyrophosphate was measured in liver microsomal preparations from rats fed 4% cholestyramine as described under Materials and Methods. Assay conditions: a) the substrate concentration was varied as indicated, while the protein concentration was 0.4 mg/ml and the incubation time 15 min; b) at 0.07 mM farnesylpyrophosphate and 0.4 mg microsomal protein/ml incubation time was varied as indicated; c) the microsomal protein concentration in the reaction mixture was varied as indicated and the reaction was performed with 0.06 mM substrate for 15 min either in the presence (●) or absence (△) of 1 mM NADPH.

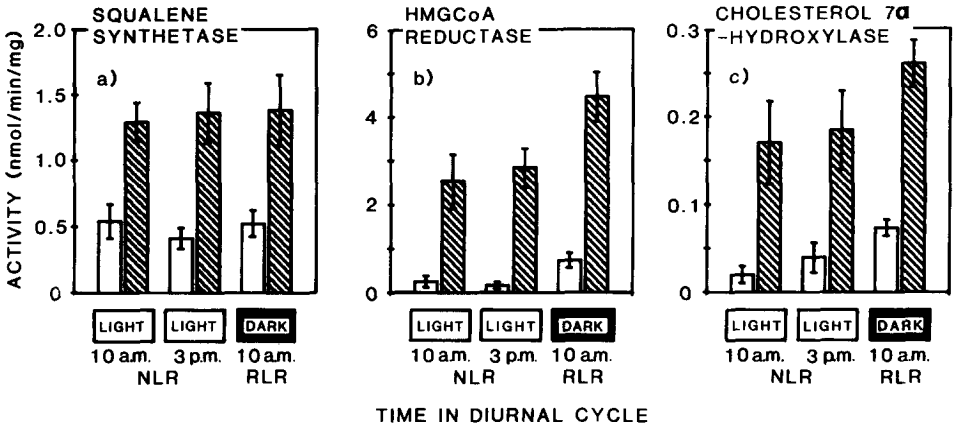

Figure 2: Effect of cholestyramine and time of killing on squalene synthetase (a), HMGCoA reductase (b) and cholesterol 7α-hydroxylase (c) activity. Rats were kept on a normal light regime (NLR) or reversed light regime (RLR), receiving either a normal diet (□) or the same supplemented with 4% (w/w) cholestyramine (▨) as indicated under Materials and Methods. (a) Squalene synthetase activity and (b) HMGCoA reductase activity were determined in duplo in microsomes prepared from the livers in the absence of KF, whereas (c) cholesterol 7α-hydroxylase activity was measured in duplo in microsomal preparations obtained in the presence of KF, as described under Materials and Methods. The average values ± S.D. for preparations from identically treated rats (n = 4) are given.

protein concentration was observed (Fig. 1c). The absolute requirement for NADPH in the reaction is shown in Fig. 1c. Similar results were obtained by Gavey and Scallen (22), working with much less active rat liver microsomal preparations.On the basis of the above we chose as standard assay conditions: farnesylpyrophosphate concentration 0.07 mM, protein concentration 0.4 mg/ml and incubation time 20 min.

Effect of cholestyramine and time of sacrifice on squalene synthetase activity: As shown in Figure 2a the enzyme activity in preparations from cholestyramine treated rats was significantly higher (average 2.8-fold) than the values obtained with the control rats, but showed no significant variation at the three different times of sacrifice.

HMGCoA reductase and cholesterol 7α-hydroxylase activity are known to display a diurnal variation with maximal activities in the dark period (1,8,23). Their activities are strongly elevated in rat by cholestyramine feeding (7,8). As is shown in Figs 2b and c, the expected differences in enzyme activities, with the highest values related to the dark period and the cholestyramine treatment, were observed in the microsomes used. These results

indicate that diurnal variations (if present) are demonstrable with our preparations.

Several other rat liver enzyme activities related to sterol metabolism were found to be elevated by cholestyramine, e.g. acetoacetyl coenzyme A synthetase (25), HMGCoA synthetase (26), sterol carrier protein 1 and 2 (27) and steroid-8-ene isomerase (28) in rat and low density lipoprotein receptor activity in dog (29). In order to exclude the possibility that the cholestyramine effect is merely the result of a general change in the metabolic state of the liver cells, we determined the activities of five control enzymes (Table 1), not directly related to sterol metabolism. The activities were measured in homogenates of the same livers used for the enzyme determinations described above. The five enzymes showed neither an effect of cholestyramine feeding nor a significant difference depending on the time of killing.

We conclude that squalene synthetase activity is elevated by cholestyramine feeding and that this effect is specific for enzymes involved in sterol metabolism. The coordinating factor(s) in this regulation is unclear. Very recently it was reported (30) that the lipid composition of the liver micro-

Table 1 Effect of cholestyramine and time of sacrifice on five enzyme activities, not directly related to sterol metabolism

Time of sacrifice	10 a.m. NLR*		3 p.m. NLR*		10 a.m. RLR*	
cholestyramine	-	+	-	+	-	+
glutamate dehydrogenase (μmol/min/mg)	1.99 ± 0.20	1.87 ± 0.18	1.82 ± 0.22	1.89 ± 0.32	2.04 ± 0.14	1.90 ± 0.11
β-hexosaminidase (nmol/min/mg)	72 ± 7	73 ± 14	62 ± 9	75 ± 21	72 ± 7	91 ± 15
NADPH cytochrome-c reductase (nmol/min/mg)	40 ± 4	30 ± 6	27 ± 5	31 ± 8	41 ± 6	46 ± 15
Catalase (nmol O_2/min/mg)	88.3 ± 6.3	93.1 ± 3.7	121.7 ± 37.5	124.9 ± 33.0	94.7 ± 9.7	93.7 ± 9.3
Acyl coenzyme A oxidase (nmol/min/mg)	7.8 ± 1.2	7.8 ± 0.9	7.5 ± 1.0	7.6 ± 1.7	7.5 ± 1.6	7.5 ± 1.3

In the rat liver cell homogenates the five enzyme activities were determined as described in the Materials and Methods section. The enzyme activities are expressed per mg of cellular protein as means ± S.D. (n = 4).
*NLR = normal light regime; RLR = reversed light regime.

somal membranes from rats fed 5% cholestyramine during 20 days did not change significantly. This suggests that the increase in squalene synthetase activity is not caused by a change in lipid composition of the microsomal membrane. We did not observe any differences in squalene synthetase activity in microsomal preparations, isolated from the same liver either in the presence or in the absence of KF (not shown), suggesting that phosphorylation/dephosphorylation does not play a role in the regulation of this activity. Possibly the increase in activity results from synthesis of new enzyme protein, as is the case with the cholestyramine induced increase of HMGCoA reductase (31). In order to study this possibility the purification of squalene synthetase, to obtain antibodies, is currently in progress.

Acknowledgements. Dr. C. Dale Poulter, Dept. of Chemistry, University of Utah, Salt Lake City, is gratefully acknowledged for providing us with pure farnesylpyrophosphate. We thank Mr. P. Koppen, Dept. of Medical Chemistry, Free University, Amsterdam, for performing the HPLC-analysis of [^{14}C]-farnesylpyrophosphate preparations. We thank Mrs M. Horsting for preparing the manuscript.

References

1. Rodwell, V.W., Nordstrom, J.L., and Mitschelen, J.J. (1976) Adv. Lipid Res. 14, 1-74.
2. Gould, R.G., and Swyryd, E.A. (1966). J. Lipid Res. 7, 698-707.
3. Slakey, L.L., Craig, M.C., Beytia, E., Briedis, A., Feldbruegge, D.H., Dugan, R.E., Qureshi, A.A., Subbarayan, C., and Porter, J.W. (1972) J. Biol. Chem. 247, 3014-3022.
4. Faust, J.R., Goldstein, J.L., and Brown, M.S. (1979) Proc. Natl. Acad. Sci. USA 76, 5018-5022.
5. Brown, M.S., and Goldstein, J.L. (1980) J. Lipid Res. 21, 505-517.
6. Huff, J.W., Gilfillan, J.L., and Hunt, V.M. (1963) Proc. Soc. Exp. Biol. Med. 114, 352-355.
7. Back, P., Hamprecht, B., and Lynen, F. (1969) Arch. Biochem. Biophys. 133, 11-21.
8. Goldfarb, S., and Pitot, H.C. (1972) J. Lipid Res. 13, 797-801.
9. Edwards, P.A., and Gould, R.G. (1972) J. Biol. Chem. 247, 1520-1524.
10. Popjàk, G., Edmond, J., Clifford, K., and Williams, O. (1969) J. Biol. Chem. 244, 1897-1918.
11. Popjàk, G. (1969) Meth. Enzymol. 15, 393-454.
12. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
13. Qureshi, A.A., Beytia, E., and Porter, J.W. (1973) J. Biol. Chem. 248, 1848-1855.
14. Philipp, B.W., and Shapiro, D.J. (1979) J. Lipid Res. 20, 588-593.
15. Goodwin, C.D., Cooper, B.W., and Margolis, S. (1982) J. Biol. Chem. 257, 4469-4472.
16. Osumi, T., and Hashimoto, T. (1978) Biochem. Biophys. Res. Commun. 83, 479-485.
17. Beaufay, H., Amar-Costesec, A., Feylmans, E.E., Thinessenpoux, D., Wibo, M., Robbi, M., and Berthet, J. (1974) J. Cell. Biol. 61, 188-200.
18. Wanders, R.J.A., Kos, M., Roest, B., Meyer, A.J., Schrakamp, G., Heymans, H.S.A., Tegelaers, W.H.H., Van den Bosch, H., Schutgens, R.B.H., and Tager, J.M. (1984) Biochem. Biophys. Res. Commun. 123, 1054-1061.

19. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
20. Krishna, G., Whitloch Jr., H.W., Feldbrugge, D.H., and Porter, J.W. (1966) Arch. Biochem. Biophys. 114, 200-215.
21. Beytia, E., Qureshi, A.A., and Porter, J.W. (1973) J. Biol. Chem. 248, 1856-1867.
22. Gavey, K.L., and Scallen, T.J. (1978) J. Biol. Chem. 253, 5470-5475.
23. Gielen, J., Van Cantfort, J., Robaye, B., and Renson, J. (1969) C.R. Acad. Sci. Paris 269, 731-732.
24. Mitropoulos, K.A., Balasubramaniam, S., Myant, N.B. (1973) Biochim. Biophys. Acta 326, 428-438.
25. Bergstrom, J.D., Robbins, K.A., and Edmond, J. (1982) Biochem. Biophys. Res. Commun. 106, 856-862.
26. Bergstrom, J.D., Wong, G.A., Edwards, P.A., and Edmond, J. (1984) J. Biol. Chem. 259, 14548-14553.
27. Morin, R.J., Brun, M.J., and Srikantaiah, M.V. (1982) Lipids 17, 507-510.
28. Yamaga, N., and Gaylor, J.L. (1978) J. Lipid Res. 19, 375-382.
29. Kovanen, P.T., Bilheimer, D.W., Goldstein, J.L., Jaramillo, J.J., and Brown, M.S. (1981) Proc. Natl. Acad. Sci. USA 78, 1194-1198.
30. Innis, S.M. (1986) Biochim. Biophys. Acta 875, 355-361.
31. Edwards, P.A., Lan, S.-F., and Fogelman, A.M. (1983) J. Biol. Chem. 258, 10219-10222.