Vol. 88, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1979 Pages 706-712

CHOLESTEROGENESIS INDUCTION IN HUMAN LYMPHOCYTES BY STEROLS EFFLUX OR PHYTOHEMAGGLUTININ STIMULATION : CELLULAR DIVISION AS THE REQUIREMENT FOR EFFICIENT CHOLESTEROL BIOSYNTHESIS IN LYMPHOCYTES

Christiane TABACIK, Monique ASTRUC, Martine LAPORTE, Bernard DESCOMPS and André CRASTES de PAULET

Unité de Recherches sur la Biochimie des Stéroïdes, INSERM - U.58 - 60, Rue de Navacelles - 34100 - MONTPELLIER - France

Received February 1,1979

SUMMARY :

It is well known that the rate of cholesterol biosynthesis in native human lymphocytes is very low but can reach a high value when the cells are stimulated either by lectins or by culture in a poor lipid medium (which induces a sterol efflux). We compared the cholesterogenesis induction by the two ways above mentioned by measuring the regulatory enzyme activity HMGCoA [a] Reductase and the real cholesterol yield (at induction time respectively 24 h and 66 h) : the HMGCoA reductase activity was very high in every case, but the cholesterol production was efficient only when the cells were stimulated to cell division. In the case of induction by sterols efflux, we observed two blocks in the biosynthetic process : 1) a shunt pathway which leads from mevalonic acid back to acetic acid, then to fatty acids, 2) a block at the lathosterol step.

These results show that HMGCoA reductase activity cannot be taken as a valuable criterion for cholesterogenesis.

1. INTRODUCTION :

For the last few years, the lymphocyte has become a very useful model for cell metabolism studies owing to the easiness with which it could be obtained in a pure form, reproducing in vitro "physiological" conditions.

It was especially used to elucidate cholesterol biosynthesis regulation in mammalian cells (1,2). Although it manifests in its native state a very low lipid metabolism (3,4), one can artificially stimulate the basal background in two different manners.

---

[a] *3-Hydroxy-3-methyl-glutaryl Coenzyme A.*

ABBREVIATIONS

HMG CoA : *3-Hydroxy-3-methyl-glutaryl Coenzyme A*
W.G.A. : *Wheat germ agglutinin*
D.L.P.S. : *delipoproteinated serum*
T.L.C. : *Thin Layer Chromatography*

0006-291X/79/100706-07$01.00/0

When the lymphocytes are maintained in a culture medium enriched with delipilated serum, there is a cholesterol efflux from the cells which respond to this deprivation by an elevation of their cholesterol biosynthesis ability (3). At the same time, takes place an increase in the high affinity LDL receptors number which reaches its maximum after 3 days of cultivation (5) ; this period is considered as the optimum time for cell cholesterol synthesis (4). But the induction of cholesterol biosynthesis can be obtained more rapidly (18-24 h) by stimulating the division of lymphocytes with lectins (4,6,7).

We compared the cholesterol biosynthesis in these two types of induction at the optimum time for each of them (about 70 h and 24 h respectively (3,6,7), by evaluating the starting impulse as HMG CoA* reductase activity, and the final result as the real amount of biosynthetized cholesterol (which is the only sterol to be used in large amounts by mammalian cells for their membrane edification) (8).

Our results reveal an impairment at the lathosterol step in the biosynthesis induced by sterol efflux and suggest that the stimulation of cellular division is a requirement for an efficient cholesterol biosynthesis.

2. MATERIALS AND METHODS :

1. MATERIALS : [2-^{14}C] sodium acetate 42 mCi/mM [2-^{14}C] DL mevalonic acid (36.5 mCi/mM) and [methyl-^{3}H] thymidine (25 Ci/mM) were prepared by C.E.A. France.

3-hydroxy-3 methyl glutaryl CoA sodium salt : was provided by P.L. Biochemicals.

Kieselgel 60 F 254 plates (DC-Alufolien) are from MERCK. For all the long run thin layer chromatographies on Kieselgel 60 F 254 plates, we used a special metallic frame to maintain the plate in a plane position. This frame was constructed in the Sciences Faculty workshop of MONTPELLIER.

Kieselgel GF plates (on glass) impregnated with 10% $AgNO_3$ come from ANACHEM.

Lathosterol : Steraloids.

Wheat germ agglutinin (W.G.A.) : Sigma, phytohemagglutinin-P (P.H.A.) : Difco.

The scanning of radioactivity was performed on a Panax Scanner.

2. METHODS : Collection and separation of lymphocytes were achieved as previously described (9), on a Ficoll-Hypaque gradient. Monocyte contamination was about 12%.

Delipoproteinated serum (DLPS) : was prepared by a 3 day ultracentrifugation of AB serum at d = 1,24 in a Beckman ultracentrifuge model L3-50, rotor 35. It was added into the culture medium RP MI-1640 to a final concentration of 20%.

---

* *3-Hydroxy-3-methyl-glutaryl Coenzyme A.*

Induction of cholesterol biosynthesis by sterol efflux : The separated lymphocytes were transferred to culture flasks, adjusted to a concentration of 4.10^6 cells per ml of medium and cultivated at 37°C for 66 h in a wet atmosphere.

The culture medium was sterile : RP MI-1640 containing 40 mM Hepes (pH 7.2), gentamicine (8 mg/100 ml) and 20% of DLPS. The viability was controlled by the tryptan blue exclusion test and was always above 95%.

Phytohemagglutinin (P.H.A.) stimulation : The separated lymphocytes were transferred to culture flasks and adjusted to a concentration of 4.10^6 cells/ml, in RP MI-1640 containing 40 mM Hepes (pH 7.2), gentamicine 8 mg/100 ml and 20% of heat inactivated human AB serum. Phytohemagglutinin (P.H.A.) was added to a final concentration of 150 µg/ml and the flasks were maintained at 37°C in a wet atmosphere for 24 h. An aliquot of the P.H.A. cultivated lymphocytes was kept for 72 h to test for [^{3}H] thymidine incorporation.

Wheat-Germ-Agglutinin (W.G.A.) stimulation : The separated lymphocytes were treated as above (P.H.A. stimulation) except that P.H.A. was replaced by W.G.A. to a concentration of 50 µg/ml (7).

Preparation of purified lymphocytes : purified lymphocytes (contamination by monocytes 2%) were prepared by Dr SERROU and his colleagues in the "Departement d'Immunologie Clinique et Expérimentale, Centre Paul Lamarque, MONTPELLIER" by the iron-carbonyle method (10).

Incubation with radioactive precursors : At the optimum induction time (66 h or 24 h respectively), the lymphocytes were separated from their culture medium by low speed centrifugation and placed in RP MI-1640 to a concentration of $3\text{-}5.10^7$ cells/3 ml. The radioactive substrat, [2-^{14}C] sodium acetate : 40 µC or [2-^{14}C]mevalonic acid : 4 µC was added in a water solution, and the cells incubated at 37°C in a normal atmosphere for 2 or 4 h*.

Isolation and identification of radioactive sterols : The incubation was stopped by adding an equal volume of 20% methanolic potassium hydroxide solution and the mixture saponified under reflux for 1 h. The nonsaponifiable material was extracted into petroleum ether (30 min. under reflux), exhaustively washed with diluted hydrochloric acid then with water and fractionated by T.L.C. on Kieselgel 60 F 254 (carbon tetrachloride, ethyl acetate 80 : 20 for 4 h continuous run).

The C-27 sterols zone was extracted from Kieselgel and acetylated (pyridine : 150 µl ; acetic anhydride : 75 µl at room temperature for 18 h).

The C-27 sterols acetate mixture was separated by T.L.C. on Kieselgel 60 F 254 (carbon tatrachloride under a continuous running for 42 h at 25°C). Under these conditions, cholesterol acetate migrates with the highest Rf, and all the C-27 sterol acetate reference samples (lathosterol, desmosterol Δ7-dehydrocholesterol) form a unique spot after migration.

The cholesterol acetate zone, and the other C-27 sterols acetates zone were extracted from Kieselgel and analyzed separately on a 10% $AgNO_3$ impregnated silicagel plate activated 30 min. at 110°C (hexane, benzene 77 : 23 for 21 h at 4°C). The lathosterol and cholesterol acetate zones were extracted from the Kieselgel, and crystallized with the corresponding cold product until constant specific radioactivity.

During all the T.L.C. separations, the radioactive metabolites were localized by comparison with authentic sterol samples and scanning of radioactivity along the plate. The radioactive metabolites were extrac-

* *we checked that the metabolite composition did not appreciably change after 2 h of incubation, following induction in the delipidated medium.*

TABLE 1 : HMG CoA Reductase activity and incorporation of [2-^{14}C] acetic acid (by 5.10^6 cells/2 h)

	MITOGENIC ACTIVATION (P.H.A. STIMULATION)			NON MITOGENIC ACTIVATION	
	ROUTINELY PREPARED LYMPHOCYTES (8)	PURIFIED LYMPHOCYTES (1) 26 H STIMULATION	PURIFIED LYMPHOCYTES (1) 42 H STIMULATION	W.G.A. STIMULATION (1)	D.L.P.S. CULTURE (4)
HMG CoA REDUCTASE ACTIVITY (PMOLES/MIN/MG /PROTEIN)	20 - 34.8 - 52.5	25	46	37	16 - 28.3 - 43
x10-^{3}DPM NONSAPONIFIABLE MATERIAL	33 - 81 - 200	70	115	29	17 - 23 - 28
% CHOLESTEROL IN NONSAPONIFIABLE MATERIAL	20 - 33.6 - 55	28	36.5	2.4	0.4 - 3.6 - 8.4
x10-^{3}DPM CHOLESTEROL	14 - 33 - 60	19.5	40.2	0.7	0.1 - 1.2 - 2.3
x10-^{3}DPM LATHOSTEROL	5.2 - 12.5 - 17.5	3.2	6.7	0.5	0.2 - 2 - 5
CHOLESTEROL / LATHOSTEROL	1.6 - 2.6 - 6	6	6	1.3	0.13 - 0.3 - 0.60

- NUMBERS IN BRACKETS REPRESENT THE NUMBER OF EXPERIMENTS
- NUMBERS UNDERLINED REPRESENT THE MEAN VALUE

ted from the Kieselgel by exhaustive elution of the desactivated gel (one drop of water) in a small chromatographic column with ethyloxide or ethyloxide, methanol 90 : 10.

Isolation of acidic metabolites : After extraction of nonsaponifiable material, the incubation mixture was acidified with hydrochloric acid 6 N under cooling and the acids extracted with petroleum ether. The acid extract was identified by T.L.C. (Kieselgel Merck - one run in hexane, ether, acetic acid - 70 : 20 : 1).

Measurements of HMG CoA reductase activity : These measurements were achieved according to the technique previously described (9).

RESULTS :

First, if we consider the HMG CoA reductase activity (which is known to be regulatory of the whole process) we found for the P.H.A. activation a mean value which is only a little higher than that obtained with the sterol efflux induction, whereas the incorporation of acetate into nonsaponifiable material and especially cholesterol is considerably higher (cf. table 1) :

- The whole neutral lipids biosynthesis is much higher in P.H.A. induction than in sterol efflux induction (2-6 times for the wholenonsaponifiable

TABLE 2 : Incorporation of [2-^{14}C] mevalonic acid (by 5.10^6 cells/2 h)

	PHA STIMULATION*	WGA STIMULATION	DLPS CULTURE
NONSAPONIFIABLE MATERIAL x10-^{3}DPM	20	2	0.6
ACIDIC FRACTION x10-^{3}DPM	92	10	3.8

* 2 EXPERIMENTS.

material, 20-100 times for the C-27 sterols (cholesterol + lathosterol = 30% of the nonsaponifiable material).

- The main sterol synthetized in P.H.A. induction is cholesterol, whereas the main sterol obtained in sterol efflux induction is lathosterol (cf. the last line in table 1). No other sterols have been identified in comparable amount.
- The cholesterol production in lymphocytes could not be attributed to monocytes as is evident from the experiment performed with purified lymphocytes (table 1, column 2). These results are not in agreement with those published recently concerning cells stimulated by sterol efflux (11).
- Lymphocytes activated with W.G.A. have a high H.M.G. CoA reductase activity (similar to the value obtained with P.H.A.), but produce only traces of cholesterol (which does not agree with the results of Pratt) (7).

In several incubation experiments of [2-^{14}C] mevalonic acid with lymphocytes activated either with P.H.A. or by sterol efflux, we isolated radioactive acids in higher yield than in nonsaponifiable material, even in P.H.A. stimulation experiments (Table 2). Only traces of cholesterol were obtained. ($\sim$ 1 % of nonsaponifiable material).

Thus pure lymphocytes (without monocytes) do synthezize cholesterol and P.H.A. activation leads to increased cholesterogenesis, whereas W.G.A. or D.L.P.S. activation result in very poor sterols formation, yielding mainly lathosterol.

DISCUSSION :

From the above results, it is clear that HMG CoA reductase activity can not be taken in any circumstance as a valuable criterion of cholestero-

genesis. It is known from Fogelman et al. (12) that a shunt pathway is able to turn off HMG CoA from cholesterol synthesis towards fatty acids synthesis : any HMG CoA formed does not necessarily produce sterols.

This shunt occurs at the prenyl-transferase step and leads from mevalonic acid back to sodium acetate which can be used as a material for fatty acid synthesis. We can observe that, even with PHA stimulated lymphocytes, [2-^{14}C] mevalonic acid does not yield cholesterol (or only as traces amounts) but produces quantities of fatty acids.

Moreover any mevalonic lactone molecule which has "escaped" the shunt does not necessarily lead to cholesterol. Radioactive lathosterol has been isolated in all the incubations of [2-^{14}C] sodium acetate with activated lymphocytes and it is the main sterol metabolite of lymphocytes to be induced by the delipidated medium (sterol efflux). As we have not detected a notable amount of Δ5,7 cholestadienol, we can conclude to an impairment of the biosynthesis at the lathosterol dehydrogenation step. This could be due to a lack of enzyme, or of sterol carrier protein, or of cytochrome b 5 which is involved in this step (13).

From the comparison of the two ways of lymphocyte cholesterogenesis induction (P.H.A. stimulation and sterol efflux), it appears that only one of them (that which induces mitoses : P.H.A.) is really efficient in cholesterol production : this can suggest that efficient cholesterogenesis in lymphocytes is related to cellular division.

ACKNOWLEDGEMENTS :

We are grateful to B. Serrou and C. Thierry, Montpellier, for their excellent collaboration in the lymphocyte preparation. This work was supported by Contract ATP n° 40-76-72 from the Institut National de la Santé et de la Recherche Médicale.

REFERENCES :

1. Williams C.D. and Avigan J. (1972) Biochim. Biophys. Acta, 260, 413-423.
2. Goldstein J.L. and Brown M.S. (1977) Metabolism, 26 n° 11, 1257-1275.
3. Fogelman A.M., Seager J., Edwards P.A. and Popjak G. (1977) J. Biol. Chem., 252, n° 2, 644-659.
4. Tabacik C., Astruc M., Laporte M., Descomps B. and Crastes de Paulet A. To be published
5. Ho Y.K., Brown M.S., Bilheimer D.W. and Goldstein J.L. (1976) J. Clin. Invest., 58, 1465-1474.
6. Chen H.W., Heiniger H.J. and Kandutsch A.A. (1975) Proc. Nat. Acad. sci. U.S.A. 72, n° 5, 1950-1954.
7. Pratt H.P., FITZGERALD P.A. and Saxon A. (1977) Cell. Immunol., 32, 160-170.
8. Demel R.A. and Kruyff B. (1976) Biochim. Biophys. Acta, 457, 109-132.

9. Laporte M., Astruc M., Tabacik C., Descomps B. and Crastes de Paulet A. (1977) Febs Letters, 86, 225-229.
10. Bertoglio J., Thierry C., Flores G., Boucharel C. et Serrou B. (1977) Clin. Exp. Immunol., 27, 172-177.
11. Fogelman A.M., Seager J., Edwards P.A., Hokom M. and Popjak G. (1977) Biochem. Biophys. Res. Comm. 76, 167-173.
12. Fogelman A.M., Edmond J. and Popjak G. (1975) J. Biol. Chem., 250 n° 5, 1771-1775.
13. Reddy V.R., Kuppler D. and Caspi E. (1977) J. Biol. Chem., 252 n° 9, 2797-2801.