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A NITROXIDE-STEROL DERIVATIVE POTENTLY MODIFIES CHOLESTEROL BIOSYNTHESIS BY NORMAL AND NEOPLASTIC GUINEA PIG LYMPHOCYTES

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Summary

Leukemic guinea pig lymphocytes (L_2 C) synthesise cholesterol in vitro at a forty-fold greater rate than normal cells. Equilibration (18 h) with lecithin or lecithin-cholesterol liposomes, respectively, enhances or suppresses sterol manufacture by normal lymphocytes but does not influence sterol production by L_2 C cells. In contrast, $> 5 \cdot 10^9$ molecules/cell of a nitroxide-derivative of androstane, (17 β-hydroxy-4′,4′-dimethylspiro [5 α-androstan-3,2′-oxazolidin]-3′-yloxyl), commonly used as a membrane spin-probe, drastically inhibit sterol production by both normal and leukemic cells (maximum within 2 h). At $< 5 \cdot 10^9$ molecules/cell, this sterol stimulates cholesterol synthesis. 25-Hydroxycholesterol at low concentrations also stimulates sterol manufacture, whereas high concentrations are also inhibitory in both cell types.

The cells of some mouse [1,2] and guinea pig [3] lymphocytic leukemias synthesize cholesterol at much greater rates than normal lymphocytes and do not reduce sterol production normally upon cholesterol feeding of the host animal. These leukemias thus exhibit a metabolic defect characteristic of hepatomas in vivo [3] i.e. impaired regulation of cholesterol biosynthesis due to deficient regulation of the relative synthesis and degradation [3] of 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34) after sterol administration. The mechanisms involved in the sterol-induced suppression of hydroxymethylglutaryl-CoA reductase biosynthesis have been recently clarified by Kandutsch and Chen [4,5], whose in vitro experiments show that certain oxidation products of cholesterol, e.g.

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25-hydroxycholesterol, 22-hydroxycholesterol, 20-hydroxycholesterol, 7-hydroxycholesterol and 7-ketocholesterol, rather than cholesterol itself, regulate sterol biosynthesis at the hydroxymethylglutaryl-CoA reductase level. The inhibitory sterols are active at concentrations between $5 \cdot 10^{-8}$ M and $5 \cdot 10^{-7}$ M and, in L-cells, can cause cell killing by inhibition of sterol biosynthesis [6].

The fact that several sterically dissimilar sterols, all more polar than cholesterol, suppress the hydroxymethylglutaryl-CoA reductase activity, suggests the possibility that this endoplasmic reticulum enzyme is regulated by the state of its membrane lipid environment. Since this state can be monitored [7] and modified [8] by the nitroxide-sterol derivative 17β-hydroxy-4′,4′-dimethylspiro [5α-androstan-3,2′-oxazolidin]-3′-yloxyl (androstane spin label), we have compared the action of this compound with that of cholesterol and 25-hydroxycholesterol on the sterol biosynthesis of normal guinea pig lymphocytes and L_2 C leukemia cells in vitro.

17β-Hydroxy-4′,4′-dimethylspiro [5α-androstan-3,2′-oxazolidin]-3′-yloxyl was purchased from Syva Corp. (Palo Alto, Calif), 5-cholesten-3β-25-diol (25-hydroxycholesterol) from Steraloids Inc. (Pawling, N.Y.), egg lecithin from Lipid Products (South Nutfield, England), cholesterol (analytical grade) from Sigma Chemical Co. (St. Louis, Mo.), Hank's Balanced Salt Solution, HEPES buffer (4-(hydroxymethyl)-1-piperazinyl-ethane-2-sulfate), phosphate-buffered saline from Gibco (Grand Island, N.Y.), minimum essential medium with Earle's salt from Associated Biometric Systems, Inc. (Buffalo, N.Y.), penicillin from Squibb (New York, N.Y.), streptomycin from E. Lilly and Co. (Indianapolis, Ind.), Ficoll^R from (Pharmacia, Inc., N.Y.), Hypaque^R from Winthrop Lab. (New York, N.Y.), Triton X-100^R from Sigma Chemical Co. (St. Louis, Mo.), and [1-^{14}C] acetate (57.8 Ci/mol) and Liquifluor^R from New England Nuclear (Cambridge, Mass.). All other reagents were of analytical grade.

The L_2 C leukemia, which arose spontaneously in a strain 2 guinea pig [9] and which has been serially passaged in syngeneic or semisyngeneic guinea pigs [10], was used in this study. This leukemic cell has been recently characterized as a B cell lymphoblast [11]. F_1 (strain 2 × Hartley) guinea pigs were inoculated subcutaneously with $5 \cdot 10^7$ viable L_2 C cells. 10 to 13 days later, when the guinea pigs became leukemic (peripheral white count $> 300\,000/mm^3$) the animals were lightly anesthetized and exanguinated by cardiac puncture. L_2 C cells were purified from heparinized peripheral blood or from a minced and strained spleen preparation by Ficoll-Hypaque gradient centrifugation [12]. Normal lymphocytes were similarly prepared from a mixture of spleen and lymph modes from F_1 guinea pigs by mincing, straining, and Ficoll-Hypaque gradient centrifugation. Macrophages were removed by adherence to plastic petri dishes for 20 min in the presence of 5% heat-inactivated fetal calf serum. The normal and leukemic lymphocytes were then washed 5 times in Hanks' balanced salt solution, once in minimal essential medium, 15 mM HEPES, pH 7.4, 100 units penicillin/ml, 0.1 mg streptomycin/ml (1000 rev./min 10 min, 4° C) and used either immediately, or after overnight storage in incubation medium as stated.

To prepare lecithin or lecithin-cholesterol liposomes (cholesterol:phos-

pholipid molar ratios 0.5, 1.0, 1.5 and 2.0) we mixed stock solution of the lipids (in chloroform/methanol; 2:1; v:v), dried first under a stream of N_2 and then for 20 min in vacuo. After addition of minimal essential medium, 15 mM HEPES pH 7.4, we sonicated for 45 min at 45°C (Sonic Dismembranator, Quigley, Rochester, N.Y.) power step 30. Liposome phospholipid was adjusted to 3 mM after phosphorus determination [13]. Cholesterol was determined as in [14].

Stock solutions of 10^{-4} M androstane spin label or 25-hydroxycholesterol were prepared by sonication of vacuum-dried sterol in minimal essential medium, 15mM HEPES, pH 7.4. Both if these sterols are water soluble up to 10^{-4} M. [1-^{14}C]Acetate, dried in vacuo from ethanolic solution was dissolved in phosphate buffered saline, pH 7.4, containing cold acetate, to obtain the desired specific radioactivity.

For [1-^{14}C]acetate incorporation into cellular lipids after exposure of cells to lecithin or lecithin-cholesterol liposomes, $\approx 2 \cdot 10^6$ cells were equilibrated with liposomes (0.3 mM lipid phosphate; cholesterol:phospholipid ratio 0—2) for 16 h at 37° C, before addition of 10 μCi [1-^{14}C]acetate (final acetate concentration 0.66 mM; volume 3 ml), and incubation continued for up to 4 h at 37° C. Controls were equilibrated with minimal essential medium buffer instead of liposomes. For experiments with androstane spin label and 25-hydroxycholesterol, the preincubation was omitted and the [1-^{14}C]acetate (18 μCi in 2 ml; acetate 0.4 mM) was added together with appropriate amounts of these sterols in buffer solution. Controls were treated identically save for addition of sterol. After incubation with labeled acetate, the viable cells were counted by Trypan Blue exclusion [15]. To determine incorporation of labeled acetate, cells were harvested using glass filters (93 LAH, Reeve Angel, Clifton, N.J.) and washed four times with 10 ml of iced phosphate buffered saline, pH 7.4. Cholesterol was collected by saponification and extraction with petroleum ether (b.p. = 38.5—50° C) [15]. The aqueous phase was brought to pH<3, with conc. HCl and extracted with 15 ml petroleum ether. The ^{14}C-labeled fatty acids (from the aqueous phase) and the ^{14}C-labeled cholesterol were then measured using a Packard Tricarb (Model 3320) Scintillation Spectrometer, using toluene, Triton X-100 and Liquifluor (67:33:4.2; v:v:v) as scintillation mixture. After incubation with androstane spin label or 25-hydroxycholesterol, the cells, deposited on glass filters and washed as before, were disrupted using 0.5 ml 0.1% dodecylsulfate and their lipids extracted and washed as in ref. 17. After drying the organic phase, this was extracted with 1.0 ml acetone/ethanol (1:1; v:v) and the cholesterol precipitated with 0.5% digitonin in 50% ethanol. The digitonide was then washed twice with 1.0 ml acetone and dissolved in 0.5 ml methanol before radioactivity assay. Each sample was in duplicate.

L_2 C cells manufacture cholesterol at a rate more than 40-fold greater than normal cells in vitro, namely $(28 \pm 1) \cdot 10^5$ cpm/10^9 viable cells/h (9 experiments), compared with $(0.65 \pm 0.12) \cdot 10^5$ cpm/10^9 viable cell/h (12 experiments). Also, as shown in Fig. 1, while normal lymphocytes increase cholesterol biosynthesis upon exposure to cholesterol free liposomes and decrease it after equilibration with cholesterol-enriched liposomes, the leukemia cells are not significantly responsive to these environmental variables.

In neither normal nor L_2 C lymphocytes is fatty acid biosynthesis significantly altered by exposure of cells to lecithin or lecithin:cholesterol liposome. Viability dropped from 80—90% to 70—80% during overnight incubation. Our in vitro experiments thus support Siperstein's in vivo observations [3].

In contrast to cholesterol, androstane spin label and 25-hydroxycholesterol, when presented as aqueous solutions, modify cholesterol biosynthesis within a few hours of incubation, during which time cell viability remains 80—90%. This phenomenon is illustrated in Fig. 2. When lymphoid cells are exposed to $2 \cdot 10^8$—$6 \cdot 10^9$ molecules of androstane spin label or 25-hydroxycholesterol per cell, sterol production is increased by up to 43% in normal cells and up to 15% or 11%, respectively, in L_2 C cells. Although the percentage stimulation is greater in normal cells, the absolute increase in biosynthesis is 20-fold greater in L_2 C cells.

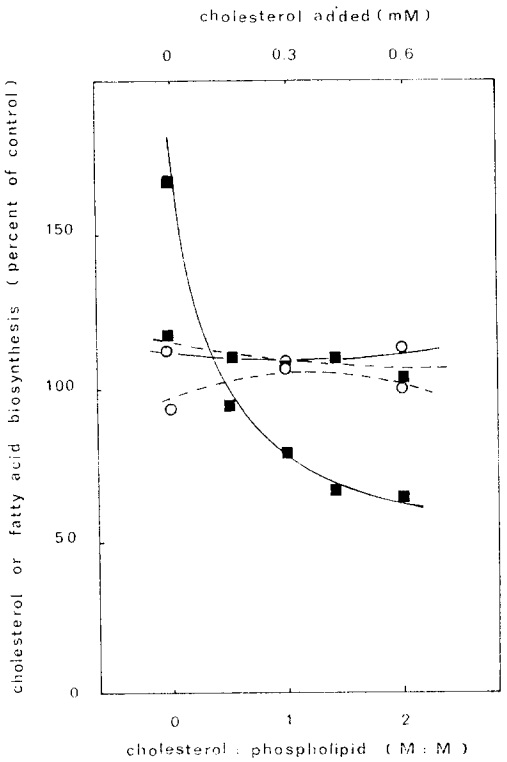

Fig. 1. In vitro effect of co-incubation of normal and leukemic guinea pig lymphocytes with lecithin or cholesterol/lecithin liposomes on cholesterol and fatty acid biosynthesis from [1-^{14}C]acetate. Cells preincubated for 16 h at 37° C with liposomes, before assay of acetate incorporation. Data expressed in terms of viable (Trypan blue-excluding) cells. The biosynthetic rates after equilibration without liposomes has been set at 100%. This corresponds to $0.6 \cdot 10^5$ cpm/10^9 cells/h and $15.5 \cdot 10^5$ cpm/10^9 cells/h for cholesterol biosynthesis by normal and L_2 C cells respectively, and $0.6 \cdot 10^5$ cpm/10^9 cells/h and $40 \cdot 10^5$ cpm/10^9 cells/h for fatty acid biosynthesis in normal and L_2 C cells respectively. The cholesterol to phospholipid ratio of the incubated liposomes is indicated along the abscissa. ■——■, cholesterol, normal lymphocytes; ●——●, cholesterol-L_2 C; ■– -- –■, fatty acid, normal lymphocytes; ●– -- --●, fatty acid-L_2 C.

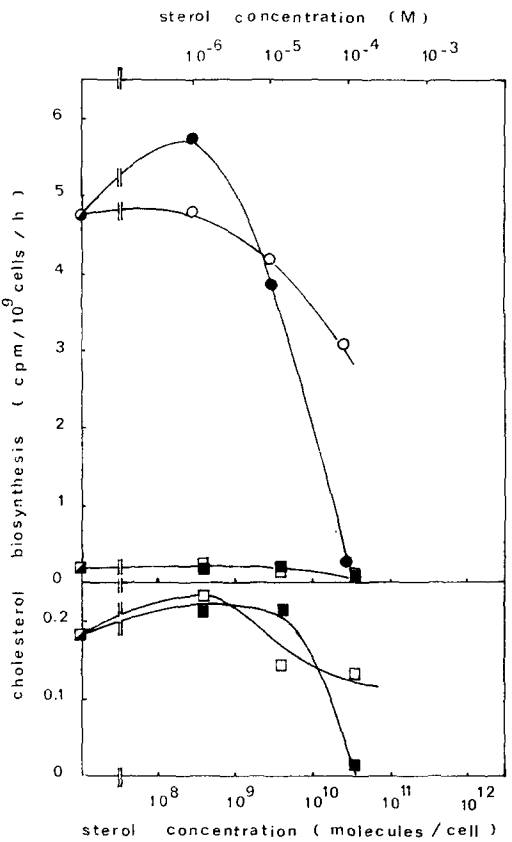

Fig. 2. Effect of androstane spin label and 25-hydroxycholesterol on sterol biosynthesis by normal and leukemic guinea pig lymphocytes. Upper panel: L_2 C cells and normal cells. Lower panel: normal cells (ordinate expanded 10-fold). ●—●: androstane spin label, L_2 C; ■—■, androstane spin label, normal. ○—○, 25-hydroxycholesterol, L_2 C; □—□, 25-hydroxycholesterol, normal.

When lymphoid cells are exposed to levels of androstane spin label or 25-hydroxycholesterol greater than $5 \cdot 10^9$ molecules/cell, cholesterol biosynthesis is suppressed. However, with androstane spin label one observed 90% inhibition between $5 \cdot 10^{10}$—$5 \cdot 10^{11}$ molecules/cell, whereas, the effect is only 30—50% with 25-hydroxycholesterol over the same concentration range.

Kinetic studies show that with 25-hydroxycholesterol there is either no effect, or slight activation of sterol biosynthesis after 2 h, whereas with androstane spin label, inhibition is already maximal at this time.

Androstane spin label is widely used as a probe for the behavior of cholesterol in biomembranes [7]. However, our data show that, with respect to its action on cholesterol biosynthesis by normal and leukemic guinea pig lymphocytes in vitro, androstane spin label simulates 25-hydroxycholesterol rather than cholesterol per se. In fact, androstane spin label

inhibits the massive cholesterol production of the leukemic lymphocytes even more effectively than 25-hydroxycholesterol, a known potent inhibitor of sterol biosynthesis [4,5], whereas cholesterol is ineffective. Androstane spin label also qualitatively simulates 25-hydroxycholesterol in that it stimulates sterol manufacture when presented at low concentrations.

The stimulatory and inhibitory phases of androstane spin label action occur in the same ranges of sterol/cell ratio as induce stabilization and labilization, respectively, of erythrocytes against osmotic stress [8]. We suspect, therefore, that the action of androstane spin label and 25-hydroxycholesterol on sterol biosynthesis may involve state modifications of the membranes bearing hydroxymethylglutaryl-CoA reductase.

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References

1 Chen, H.W., Kandutsch, A.A., Heinger, H.J. and Meier, H. (1973) Cancer Res. 33, 2774—2778
2 Chen, H.W. and Heiniger, H.J. (1974) Cancer Res. 34, 1304—1307
3 Siperstein, M.D. (1970) in Current Topics in Cellular Regulation (Horecker, B.L. and Stadtman, E.R., eds), pp. 65—100, Academic Press, New York
4 Kandutsch, A.A. and Chen, H.W. (1973) J. Biol. Chem. 248, 8408—8417
5 Kandutsch, A.A. and Chen, H.W. (1974) J. Biol. Chem. 249, 6057—6061
6 Chen, H.W., Kandutsch, A.A. and Waymouth, C. (1974) Nature 251, 419—421
7 Keith, A.D., Sharnoff, M. and Cohn, G.E. (1973) Biochim. Biophys. Acta 300, 379—419
8 Bieri, V.G., Wallach, D.F.H. and Lin, P.S. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4797—4801
9 Congdon, C.D. and Lorenz, E. (1954) Am. J. Pathol. 30, 337—359
10 Gross, L. and Dreyfuss, Y. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3550—3554
11 Shevach, E.M., Ellman, L., Davie, J.M. and Green, I. (1972) Blood 39, 1—12
12 Boyum, A. (1974) Tissue Antigens 4, 269—274
13 Lowry, O.H., Roberts, N.R., Leiner, K.Y., Wu, M.L. and Farr, A.L. (1954) J. Biol. Chem. 207, 1—17
14 Parekh, A.C. and Jung, D.H. (1970) Anal. Chem. 62, 1423—1427
15 Phillips, H.J. (1973) in Tissue Culture, Methods and Applications (Kruse, P.F. and Patterson, M.K. eds), pp. 405—408, Academic Press, New York
16 Conner, R.L., Mallory, F.B., Landrey, J.R. and Iyengar, W.L. (1969) J. Biol. Chem. 244, 2325—2333
17 Folch, L., Lees, M. and Stanley, G.H.S. (1957) J. Biol. Chem. 278, 497—509