Cholesterol Homeostasis

MODULATION BY AMPHIPHILES*

(Received for publication, August 25, 1994, and in revised form, September 30, 1994)

Yvonne Lange‡ and Theodore L. Steck¶

From the ‡Departments of Pathology and Biochemistry, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612 and the ¶Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

Diverse amphiphiles act on cellular cholesterol metabolism as if signaling regulatory sites. One class (oxysterols) mimics the homeostatic effects of excess cell cholesterol, inhibiting cholesterol biosynthesis and stimulating plasma membrane cholesterol esterification. A second class of amphiphiles has effects precisely opposite to the oxysterols, i.e. they immediately inhibit plasma membrane cholesterol esterification and progressively induce 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and cholesterol biosynthesis. This second class of agents includes steroids, hydrophobic amines, phenothiazines, ionophores, colchicine, cytchalasins, and lysophosphatidates, most of which interact with P-glycoproteins.

These data support a general hypothesis describing cellular cholesterol homeostasis. (a) Proteins regulating sterol metabolism are embedded in intracellular membranes where their activities are governed by the local level of cholesterol. (b) Excess plasma membrane and lysosomal cholesterol circulates through those intracellular membranes and sets the homeostatic activities therein. (c) The two classes of agents mentioned above affect cholesterol homeostasis by increasing or decreasing, respectively, the ambient level of cholesterol at the sites of regulation.

Cholesterol regulates its own abundance in animal cells through feedback control of several homeostatic activities. These include accretion by ingestion, biosynthesis, and cholesterol ester hydrolysis; and consumption by cholesterol esterification, secretion, and transformation into sterols, bile acids, and lipoproteins (1, 2). Although the sitter that mediate these activities reside in cytoplasmic organelles, almost all unesterified cell cholesterol is confined to the plasma membrane (3–5). An unanswered question is how the intracellular sites gauge and regulate the pool in the plasma membrane.

Recent evidence suggests the existence of a sensor mechanism by which plasma membrane cholesterol exceeding a threshold is transported to intracellular sites for conversion to esters and steroids (6–12). In this regard, excess cell cholesterol can be mimicked by certain exogenous oxysterols (1, 2), which we shall refer to as class 1 agents. In particular, 25-HC (7) stimulates cholesterol esterification (6, 13–15) and conversion to steroids (13, 16); it also stimulates the secretion of cholesterol from liver cells as LDL (14) and down-regulates HMG-CoA reductase activity (17). Like cholesterol, oxysterols inhibit the expression of genes coding for HMG-CoA reductase and LDL receptors (1, 2, 15).

A second set of compounds, which we shall call class 2, has effects opposite to those of excess cholesterol; these agents inhibit cholesterol esterification, stimulate cholesterol biosynthesis, and counter the action of oxysterols. The first members of this group to be described were steroids such as progesterone (14, 18–21, 44) and hydrophobic amines like imipramine and the steroid U18666A (15, 22). We now show that class 2 contains a wide variety of other amphiphiles, most of which interact with P-glycoproteins. Furthermore, the reciprocal antagonism of class 1 and class 2 agents supports a hypothesis for the mechanism underlying intracellular cholesterol homeostasis.

RESULTS

Fig. 1 shows that the esterification of cholesterol in intact cells is inhibited by a variety of amphiphilic agents. Similar effects were also obtained with the agents employed in Table I as well as with lysophosphatidylserine and lysophosphatidylethanolamine (not shown). This inhibition was immediate in all cases and was reversed by washing when tested with progesterone, monensin, imipramine, and chlorpromazine. Blocking protein synthesis with 25 μg cycloheximide did not prevent the inhibition of esterification by any agent tested (nigerinic, lysophosphatidylcholine, monensin, progesterone, chloroquine, and trifluoperazine). The inhibition was antagonized by 25-HC (Fig. 2) as well as 7-ketocholesterol (not shown).

As illustrated in Table I, the inhibitors of cholesterol esterification stimulate plasma sterol biosynthesis and HMG-CoA reductase activity. Where tested (progesterone, monensin, and lysophosphatidylcholine), the stimulation accelerated characteristically over a period of up to 20 h. Stimulation by progesterone was blocked by cycloheximide. None of several agents tested (progesterone, monensin, lysophosphatidylcholine, nigerinic, and trifluoperazine) had an appreciable effect on HMG-CoA reductase activity when added to homogenates. The effect of these agents on cholesterol biosynthesis in intact cells was opposed by 25-HC and 7-ketocholesterol (not shown). These are the attributes of class 2 agents.

It has been shown previously that the utilization of plasma membrane cholesterol for esterification and steroidogenesis is inhibited by certain compounds which perturb calmodulin (namely trifluoperazine), acidic vacuoles (chloroquine and nigerinic), microtubules (colchicine), and microfilaments (cytchalasins) (7). That these inhibitors are class 2 agents is now suggested by the fact that they also stimulate cholesterol biosynthesis and HMG-CoA reductase activity (Table I). Furthermore, the inhibition of cholesterol esterification by these agents was opposed by 25-HC (Fig. 2) and 7-ketocholesterol (not shown); this should not be the case if their mode of action was on the subcellular structures originally proposed (7).
FIG. 1. Class 2 agents inhibit cholesterol esterification. Confluent monolayers of cultured rat hepatoma cells, line FUSAH (11), were washed and suspended in 5 ml of 150 mM NaCl, 5 mM NaF (pH 7.5), 5 mM glucose. A total of 6–10 aliquots were dispensed from each flask (containing ~60 μg of cell cholesterol) and preincubated for 5 min at 37°C with class 2 compounds in Me2SO or ethanol (<1% final). [3H]Oleic acid (0.04 μCi) was added for an incubation of 10 min at 37°C. The incorporation of label into cholesteryl esters was determined (23). The values, normalized as dpm/pg cell cholesterol, are means of duplicates that agreed to within 10%. Parallel flasks were washed and suspended in 5% glucose. A total of 150 μCi/μl of cell cholesterol, while those in class 2 inhibited it (Table 1). The values, expressed per mg protein, are means of duplicates which agreed to within 10%. Parallel flasks were incubated for 15–16 h at 37°C. To determine sterol synthesis in vivo, 10 μCi [3H]acetate/ml was added and the flasks incubated for 1 h. The incorporation of label into total sterols was determined (11, 21); ~1% of total label was incorporated in the controls. Values are averages of duplicate samples or replicate experiments. Abscissa, fraction of the following maximal concentrations. Panel A: ○, 37 μM progesterone; △, 37 μM testosterone; ▽, 37 μM estradiol. Panel B: ○, 32 μM progesterone; ◊, 46 μM lysophosphatidylcholine; ◼, 3 μM monensin.

**TABLE I**

The effect of class 2 agents on sterol biosynthesis and HMG-CoA reductase activity

Subconfluent human fibroblasts (23) were given fresh growth medium containing 10% fetal bovine serum plus agents in Me2SO or ethanol (<1% final) and preincubated for 15–16 h at 37°C. To determine sterol synthesis in vivo, 10 μCi [3H]acetate/ml was added and the flasks incubated for 1 h. The incorporation of label into total sterols (desmosterol, 7-dehydrocholesterol, lanosterol, plus cholesterol) was determined (23). The values, normalized as dpm/pg cell cholesterol, are means of duplicates which agreed to within 10%. Parallel flasks were also extracted with 1% Kyr EOB (42) in a buffer containing 50 mM sodium fluoride and HMG-CoA reductase activity determined (43). The values, expressed per mg protein, are means of duplicates that agreed to within 5%. In the absence of agents, values ranged from 1000 to 4000 dpm/h/mg protein.

| Agent                        | Concentration | Relative activity |
|------------------------------|---------------|------------------|
|                              | μM            | [3H]Sterol       | HMG-CoA reductase |
| None                         | 0             | 1                | 1                |
| Lysophosphatidylcholine      | 42            | 2                | 4                |
| Colchicine                   | 80            | 12               | 5                |
| Progesterone                 | 10            | 20               | 14               |
| Nigeric                      | 3             | 22               | 14               |
| Chloroquine                  | 80            | 32               | 44               |
| Trifluoperazine              | 20            | 37               | 67               |
| Monensin                     | 0.3           | 85               | 44               |
| Imipramine                   | 80            | 85               | 65               |

Class 1 agents stimulated the esterification of plasma membrane [3H]cholesterol, while those in class 2 inhibited it (Table II, experiments A and B). Experiment C in Table II shows that while 25-HC does not appreciably stimulate esterification in cells fed LDL, presumably because feeding has already stimulated them, it reverses the inhibition of esterification by agents in class 2.

**TABLE II**

Effect of agents on esterification of plasma membrane [3H]cholesterol

Cultured hepatoma cells were labeled in suspension with [3H]cholesterol (11). Aliquots were prepared and incubated with agents as in Fig. 1. The incorporation of label into cholesteryl esters was determined following a 40-min incubation at 37°C (11). In Experiment A, cells were grown for 16 h in medium containing lipoprotein-deficient serum to enhance basal HMG-CoA-reductase activity. Conversely, in experiments B and C, cells were grown in full medium (11). Values are means of duplicate determinations that agreed to within 10%. In the untreated controls, ~2,000 dpm or ~1% of cell [3H] was incorporated into esters.

| Expt. | Agents                        | Concentration | [3H]Cholesterol esterification |
|-------|-------------------------------|---------------|--------------------------------|
| A     | 25-Hydroxycholesterol         | 40            | 197                            |
| B     | Imipramine                    | 100           | 31                             |
|       | Trifluoperazine                | 20            | 30                             |
|       | Progesterone                   | 6             | 29                             |
|       | Monensin                      | 1             | 23                             |
|       | Lysophosphatidylcholine        | 30            | 15                             |
|       | Nigeric                       | 3             | 9                              |
| C     | 25-Hydroxycholesterol         | 60            | 116                            |
|       | Progesterone                   | 6             | 20                             |
|       | 25-HC + progesterone          | 60            | 40                             |
|       | Monensin                      | 1             | 18                             |
|       | 25-HC + monensin              | 60            | 42                             |

DISCUSSION

A coherent body of evidence suggests that the compounds studied here act upon the transfer of plasma membrane cholesterol to cytoplasmic membranes. First, it is clear that cholesterol moves among cellular membranes, although the mechanism mediating this process is obscure (22). Cholesterol liberated from LDL in lysosomes, for example, is promptly and preferentially transported to the plasma membrane (22). The same is true of both the cholesterol and its precursors synthesized in the ER (23). Perhaps it is this rapid export from the ER that keeps nascent cholesterol
Regulation of Cholesterol Homeostasis

from interacting with the regulatory sites in the same membranes.) That exogenous \(^{3}H\) sterols are transferred from the plasma membrane to the ER and mitochondria is demonstrated by their esterification, 7-0-hydroxylation, conversion to steroids, and, in the case of sterol intermediates, by their conversion to cholesterol (6–11, 13, 24, 25). The circulation of plasma membrane cholesterol through the cell interior may proceed at rates as high as 100% h\(^{-1}\), much faster than cholesterol ingestion, biosynthesis, and consumption (11).

Vectorial transport presumably drives the accumulation of cholesterol in the plasma membrane and its depletion from the internal membranes (4); the cholesterol content of the ER and mitochondria is so low as to be not clearly demonstrable in isolates once correction is made for contamination by the sterol-rich plasma membranes (3–5, 11, 26). As a result, the rates of esterification, 7-hydroxylation, and steroidogenesis in those organelles are limited by the delivery of exogenous cholesterol (8–11, 13, 25).

The following evidence suggests that the compounds studied here modulate the movement of plasma membrane cholesterol to internal membranes. \((a)\) 25-HC stimulates the esterification of cell surface radiocholesterol (Table II and Ref. 6). A strong stimulation of cholesterol esterification by 25-HC is also seen in cells where the plasma membrane provides the only substantial pool of substrate (15). \((b)\) Various class 2 agents inhibit both the esterification and the conversion to steroids of radiocholesterol placed in the plasma membrane (Table II and Refs. 7, 12, 13, and 21). \((c)\) Progesterone inhibits cholesterol influx by prostegesterone, incidentally, that nascent intracellular steroids could feed back negatively on their own biosynthesis by reducing the delivery of plasma membrane cholesterol to mitochondria. \((d)\) Progesterone inhibits the transfer of plasma membrane radiocholesterol to a discrete intracellular membrane compartment (21), which might serve as an intracellular transporter (4). We have found that monensin and lysophosphatidylinositol act similarly (not shown). \((e)\) The fact that lysophosphatidylethanolamines added to intact cells inhibit cholesterol esterification instantly (Fig. 1) even though they take hours to cross bilayers (27) suggests that they act at the plasma membrane. \((f)\) Progesterone does not inhibit the esterification of newly-synthesized cholesterol in the ER (21). Similarly, class 2 agents do not inhibit esterification in broken cell preparations except when the transfer of cholesterol to the fragments of ER is rate-limiting (19, 21). \((g)\) Progesterone inhibits the conversion of the late sterols in the plasma membrane to cholesterol in the ER (21). We have observed similar effects with all of the agents in Table I; they stimulate overall sterol biosynthesis while promoting the relative accumulation of precursors (not shown). These data explain the puzzling observation that progesterone blocks cholesterol biosynthesis even while stimulating HMG-CoA reductase activity (18).

Some class 2 agents have been found to trap ingested cholesterol in the lysosomes (15, 19, 20, 22). A similar phenomenon is seen in certain mutant cells (22, 28–30). The site of the genetic block is not known.

These data support the following parsimonious hypothesis for cellular cholesterol homeostasis. Intracellular circulation provides a link between the bulk of the cell cholesterol in the plasma membrane and internal homeostatic sites. In this model, plasma membrane cholesterol in excess of a threshold level is shifted by a putative sensor to the intracellular membranes, where it accumulates in proportion to its excess in the plasma membrane (9, 10). The consequent level of cholesterol in the intracellular membranes directly controls the activities of the homeostatic enzymes (7–11) and regulates the turnover of HMG-CoA reductase by modulating its phosphorylation and proteolysis (31, 32). Thus, in the short term, the steady-state excess of internalized cholesterol will be esterified or otherwise consumed. A persistent excess cholesterol in the ER would slowly down-regulate the proteins mediating sterol biosynthesis and LDL uptake (31–33). A converse response would bolster plasma membrane cholesterol when it fell below the threshold. The threshold setting of the sensor might itself be regulated, e.g. by trophic hormones, which stimulate steroidogenesis (34), or by the magnitude of the plasma membrane bilayer (10).

Oxysterols are generally thought to act at the level of gene expression (1, 2); however, this premise does not account for their immediate stimulation of plasma membrane cholesterol esterification (Table II and Fig. 2) (6, 13) nor for the failure of cycloheximide to block this stimulation. The enhanced ester formation is not simply due to esterification of 25-HC itself, although this occurs. Rather, 25-HC stimulates the esterification of cellular cholesterol (Fig. 2) (13–15) and plasma membrane \(^{3}H\) cholesterol in particular (Table II) (6). We therefore suggest that class 1 oxysterols increase the steady-state concentration of cholesterol at homeostatic loci.

Recently, the expression of HMG-CoA synthase and LDL receptors was shown to be triggered by the proteolytic release of a transcription factor precursor anchored in the ER and outer nuclear membranes (33). The proteolytic cleavage step is apparently under negative control by steroids. Exogenous oxysterols could act directly at the ER and/or by stimulating cholesterol delivery to the ER.

Class 2 agents as diverse as steroids, lysophosphatidylcholines, phenothiazines, and other hydrophobic amines all stimulated the expression of HMG-CoA reductase (Table I). There is no evidence that this array of agents interacts directly with transcription factors or acts through a common messenger pathway. Rather, we would like to consider that class 2 agents activate the relevant transcription factor precursors (33) by reducing the level of cholesterol in the ER. The argument is strongest for lysophosphatidylcholines; since they do not readily penetrate the plasma membrane so as to enter the cell (27), these agents could act by reducing the flux of plasma membrane cholesterol through the cell interior. The model predicts that all class 2 agents will similarly stimulate LDL receptor expression.

Class 1 and class 2 agents behave as reciprocal agonists and antagonists. In the simplest case, they might bind with mutual exclusion at a common site (true competition). Alternatively, they could act at separate sites along a pathway. In that case, however, agonists should not overcome antagonists as is observed in Fig. 2 and Table II (experiment C). Similarly, the evidence does not favor parallel homeostatic pathways, each susceptible to only one class of agent. An even less likely explanation for the observed antagonism would be direct physical interactions between members of the two classes (35).

The structural diversity among the agents in class 1 and, especially, class 2 suggests that they might not interact steroispecifically with a receptor. This premise is also consistent with the low potency of class 2 steroids compared to their affinity for known receptors; furthermore, these steroids affect a broad range of cells not known to have such receptors (19). Rather, these amphiphiles might partition into bilayers and alter their physical properties so as to confuse the putative sensor concerning the abundance of cholesterol (36, 37). Alternatively, the action of class 2 agents might involve a P-glycoprotein, given that those class 2 compounds tested all countered multi-drug resistance (38–40, 44). There is evidence, on the other hand, that cholesterol itself does not compete with ligands for P-glycoproteins (41).

Acknowledgments—We thank Francisco Strebel and Jin Ye for excellent technical assistance.
REFERENCES

1. Fielding, C. J., and Fielding, P. E. (1988) in Biochemistry of Lipids and Membranes (Vance, D. E., and Vance, J. E., eds) pp. 404–474, Benjamin/ Cummins, Menlo Park, CA.

2. Goldstein, J. L., and Brown, M. S. (1990) Nature 343, 420–430.

3. Lange, Y., Sevasgood, M. H., Ramos, B. V., and Steck, T. L. (1989) J. Biol. Chem. 264, 3796–3798.

4. Lisco, A. K., Xu, X. X., Shiratori, Y., and Tabas, I. (1994) J. Biol. Chem. 269, 991–996.

5. Lange, Y. (1994) J. Biol. Chem. 269, 991–996.

6. Lisco, A. K., Xu, X. X., Shiratori, Y., and Tabas, I. (1994) J. Biol. Chem. 269, 991–996.

7. Nagy, L., and Freeman, D. A. (1990) J. Biol. Chem. 265, 105–110.

8. Nagy, L., and Freeman, D. A. (1990) J. Biol. Chem. 265, 105–110.

9. Xu, X. X., and Tabas, I. (1991) J. Biol. Chem. 266, 17040–17048.

10. Okwu, A. K., Xu, X. X., Shiratori, Y., and Tabas, I. (1994) J. Biol. Chem. 269, 991–996.

11. Lange, Y., Strebel, F., and Steck, T. L. (1993) J. Biol. Chem. 268, 13838–13843.

12. Harnalla, A. S., Fern, M. I., Mattius, P., and Sotte, J. P. (1994) Biochim. Biophys. Acta 1211, 317–321.

13. Miller, S. C., and Melnykovych, G. (1984) In Lipid Res. 25, pp. 991–996.

14. Dashi, N. (1992) J. Biol. Chem. 267, 1107–1113.

15. Iida, S., Papadopoulos, V., and Hall, P. F. (1989) Endocrinology 124, 2627–2627.

16. Bell, J. J., Sargeant, T. E., and Watson, J. A. (1976) J. Biol. Chem. 251, 1745–1758.

17. Parini, S. R., Gupta, A., Sexton, R. C., Parish, E. J., and Rodney, H. (1987) J. Biol. Chem. 262, 14435–14440.

18. Butler, J. D., Blanchette-Mackie, J., Goodin, E. O'Neill, R. R., Carstea, G., Roff, C. F., Paterson, M. C., Patel, S., Comly, M. E., Cooney, A., Vanier, M. T., Brady, R. O., and Pentchev, P. G. (1992) J. Biol. Chem. 267, 23805–23805.

19. Furusho, K., Shinagawa, T., Arai, T., and Inoue, K. (1993) J. Biol. Chem. 268, 27345–27348.

20. Lange, Y. (1994) In Lipid Res. 26, 3141–3141.

21. Lisco, L., and Dahl, N. K. (1992) J. Biol. Chem. 263, 1239–1254.

22. Lange, Y., Echavarria, F., and Steck, T. L. (1991) J. Biol. Chem. 266, 21439–21443.

23. Freeman, D. A. (1989) Endocrinology 124, 2527–2534.

24. Straka, M., Junker, L. H., Zaczek, L., Zagg, D. L., Duetland, S., Eversen, G. T., and Davis, R. A. (1990) J. Biol. Chem. 265, 7145–7149.

25. Cok, S. J. (1995) Cholesterol Transport and Metabolism by Mitochondria of Steroidogenic Cells. Ph.D. dissertation, The University of Chicago.

26. Mohandas, N., Greenquist, A. C., and Hook, S. B. (1978) J. Supramol. Struct. 9, 455–458.

27. Pentchev, P. G., Comly, M. E., Kruth, H. S., Tobisu, T., Butler, J., Sokol, J., Filling-Katz, M. Quirk, J. M., Marshall, D. C., Patel, S., Vanier, M. T., and Brady, R. O. (1987) FASEB J. 1, 40–45.

28. Metherall, J. E., Ridgway, N. D., Dawson, P. A., Goldberg, J. L., and Brown, M. S. (1991) J. Biol. Chem. 266, 12734–12740.

29. Dahk, N. K., Guthel, W. G., and Lisco, L. (1993) J. Biol. Chem. 268, 16975–16985.

30. Meigs, T. E., and Simoni, M. R. (1992) J. Biol. Chem. 267, 13547–13552.

31. Omkumar, R., Darnay, B. G., and Rodwell, V. W. (1994) J. Biol. Chem. 269, 6519–6614.

32. Ding, X., Sato, R., Brown, M. S., Hua, X., and Goldberg, J. L. (1994) Cell 77, 53–62.

33. Murphy, B. D., and Silavon, S. L. (1989) Oxford Rev. Reprod. Biol. 11, 179–223.

34. Gaddum, J. H. (1957) Pharmacol. Rev. 9, 211–218.

35. Carlson, J. C., Gruber, M. Y., and Thompson, J. B. (1983) Endocrinology 113, 190–194.

36. Kodavanti, U. P., and Mohendran, D. H. (1990) Pharmacol. Rev. 42, 327–354.

37. Ford, J., and Halt, W. W. (1990) Pharmacol. Rev. 42, 155–159.

38. Teur, T. (1991) in Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells (Rimawi, M., ed) pp. 349–372, Focal Press, New York.

39. Gottesman, M. M., and Pastan, I. (1992) Annu. Rev. Biochem. 61, 885–907.

40. Saeki, T., Shimabuku, R., and Take, S. (1992) Biochim. Biophys. Acta 1107, 105–110.

41. Brown, M. S., Davis, S. E., and Goldstein, J. L. (1976) Proc. Natl. Acad. Sci. U. S. A. 76, 2162–2166.

42. Drevon, C. A., Weintraub, D. B., and Steinberg, D. (1986) J. Biol. Chem. 261, 9124–9137.

43. deBry, P. W., and Metherall, J. E. (1994) J. Biol. Chem. 269, 35A (abstr.)