review

Tracking cell cholesterol with cholesterol oxidase

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Sterols are ubiquitous and essential constituents of all eukaryotic cells, found either esterified and stored in droplet form (1), or unesterified and integrated into bilayers (2). Sterols not only play a major structural role in membranes, but serve as precursors for steroid hormones and bile acids (3). The distribution of cholesterol within the cell and how it is apportioned properly are not only of fundamental interest but of cardinal medical importance. Despite intensive study (reviewed in 4–7), the basic mechanisms of apportionment among cellular membranes of structural lipids in general and cholesterol in particular are still poorly understood. This brief review will focus on the utility of a particular probe, cholesterol oxidase.

VARIABLES THAT AFFECT THE ACTION OF CHOLESTEROL OXIDASE

Cholesterol oxidase [EC 1.1.3.6] attacks sterols at the 3β-hydroxyl position to form Δ^4-cholestenone and H_2O_2 (8). The action of cholesterol oxidase is strongly influenced by the environment of its substrate even in simple detergent solutions (9). The attack of cholesterol oxidase on mixed lipid monolayers is inhibited by increasing lateral surface pressure (10). In membranes, phospholipid composition and cholesterol content can be critical (11). The cholesterol at the cytoplasmic surface of red cell membranes is a good substrate for the enzyme under conditions where cholesterol at the external surface is barely reactive (12, 13). The cholesterol in the outer leaflet of plasma membranes in several other cell types is also poorly reactive in physiological saline but becomes a good substrate either when membrane cholesterol is increased or following exposure of the cells to glutaraldehyde or to warm, low ionic strength buffers (13). Divalent cations are more potent inhibitors of enzyme action than monovalent cations; the anion species seem indifferent, suggesting that electrolytes inhibit oxidation by contributing cations that bind anionic membrane sites. Intercalators such as decane and octanol stimulate oxidation; lysolecithin is an inhibitor (13). Both phospholipase C (14) and sphingomyelinase (15) greatly increase the susceptibility of membrane cholesterol to oxidation. The molecular basis for the conditionality of the action of cholesterol oxidase on membranes is not understood; however, a similar behavior also has been described for phospholipase A_2 where access of the enzyme to the substrate site seems to be the critical issue (16).

PROBLEMS IN USING CHOLESTEROL OXIDASE AS A PROBE

The conditionality discussed above creates a practical problem: cellular cholesterol may not be oxidized under certain conditions and yet be a good substrate under others. We have dealt with this issue by carefully optimizing and rigorously controlling key experimental variables (13). By these means, the oxidation reaction rarely fails.

One application of cholesterol oxidase, discussed below, is the delineation of plasma membrane cholesterol as that oxidized in the intact cell. However, cholesterol oxidase treatment can promote the passive permeability of the plasma membrane to small solutes such as potassium ions (17). This is of concern because such leaks could lead to osmotic swelling, cellular lysis, and the oxidation of intracellular pools. Nevertheless, in the study mentioned (17), the leakage of lactate dehydrogenase from the cytoplasm was negligible. Given this impermeability to macromolecules, cholesterol oxidase should not enter the cytoplasm.

We have observed the breakdown of cells after cholesterol oxidase treatment. Because this untoward effect follows extensive oxidation, it can be minimized by not prolonging the reaction unnecessarily. Furthermore, this hazard can be circumvented entirely by the prior fixation of the target cells with glutaraldehyde which has the added benefit of increasing the effect of

the oxidase (13). Unfortunately, the expedient of fixation cannot be applied when subcellular fractions are to be prepared from cholesterol oxidase-treated cells.

Cholesterol oxidase treatment of whole cells does not attack intracellular sterol pools. For example, sterol oxidation is no greater in unfixed cells than in cells treated with glutaraldehyde. Furthermore, the cholesterol oxidized by treating cells with the enzyme copurifies with the plasma membrane while the unoxidized fraction is recovered in other membranes (18). In addition, nascent cholesterol becomes susceptible to cholesterol oxidase in intact cells, both fixed and unfixed, only after a lag period during which it is transferred to the plasma membrane (18, 19).

We were surprised recently to find that cell breakage, whether inadvertent or intentional, need not lead to the oxidation of intracellular cholesterol. Thus, the cholesterol in the organelle membranes of fibroblasts (20) and rat hepatoma cells is not a substrate for cholesterol oxidase in homogenates. This behavior may reflect differences in the environment of the cholesterol in intracellular organelles compared to the plasma membrane, another manifestation of the conditional susceptibility of sterols to cholesterol oxidase, discussed above. In any case, it is fortunate that the hazard of cell breakdown during cholesterol oxidase treatment is offset by the resistance of the cytoplasmic membranes to the enzyme, at least in the cell types examined thus far.

Given the large fraction of cholesterol oxidized by the enzyme in intact cells (see below), it has been suggested that the treatment induces redistribution of intracellular cholesterol to the cell surface. This appears unlikely for several reasons. First, the fraction of cell cholesterol which is oxidizable is the same in unfixed cells as it is in fixed cells where redistribution of sterol is unlikely to occur. Second, the fraction of oxidizable cholesterol corresponds closely to the fraction of total cell cholesterol which can be ascribed to the plasma membrane estimated by independent methods. Finally, greater than 90% of the cholesterol in intact fibroblasts can be oxidized in about a minute, too brief a period for cholesterol to diffuse or be carried through the cytoplasm by vesicles.

KINETICS OF THE TRANSMEMBRANE MOVEMENT OF CHOLESTEROL

A variety of methods have been applied to the measurement of the rate of movement of cholesterol across membranes. Half-times for the process of from less than 3 sec (21) to more than 10 days (22) were reported (see ref. 23). It seems likely that this spread in values of five orders of magnitude reflects the techniques applied rather than the mobility of sterols in bilayers. The most rapid flip rates have been obtained using cholesterol oxidase. In particular, almost all of the cholesterol contained in sonicated unilamellar vesicles of egg phosphatidylcholine could be oxidized by cholesterol oxidase with a single time constant of about 1 min at 37°C (24). Control experiments showed that the vesicles retained their integrity and did not release their contents as a result of enzyme treatment. Given that the enzyme presumably had access only to the outer membrane leaflet, it seems that either all of the sterol was confined to this surface or, more likely, cholesterol moved across the membrane with a half-time of less than 1 min. Similarly, essentially all of the cholesterol in human erythrocytes was oxidized in a first order fashion with a half-time of less than 3 sec at 37°C (21). Lysis of the cells, monitored by the release of hemoglobin, was negligible (21). Since the rate of the reaction increased with enzyme concentration, it seemed that cholesterol flip was fast compared to the oxidation step. Ninety to 95% of the cholesterol in intact fixed human fibroblasts was attacked by the enzyme with a half-time of less than 30 sec at 37°C (25); these kinetics might also have been enzyme-limited. All of these data suggest that sterols flip rapidly across membranes.

In contrast to these studies, a more intricate application of the cholesterol oxidase technique has suggested a half-time of 1–2 h for the transmembrane movement of cholesterol in red cells (23); this value is three orders of magnitude slower than those given above. In this study, intact erythrocytes were pulsed with [^{3}H]cholesterol; aliquots were then subjected to a series of brief cholesterol oxidase treatments to establish the time course of change in the specific activity of the [^{3}H]cholestenone product. One way to reconcile these data with those described above is to postulate that the exogenous radiolabeled cholesterol probe became integrated into the bilayer with difficulty, so that the observed kinetics reflected this slow process rather than flip-flop. Since the assay procedure involved an exposure to the enzyme of only 20 sec, during which time only 3% of the cholesterol was oxidized, a skewed, nonrepresentative sampling of the exogenous [^{3}H]cholesterol might also have complicated this analysis.

In deciding between estimates of flip times of a few seconds or less (21) versus hours (23) to days (22), we can have recourse to studies of the diffusion of other weak amphipaths across bilayers. Aliphatic alcohols, for example, generally cross red cell membranes on a time scale of milliseconds (26). Certain biological lipids also appear to cross membranes within seconds

or minutes; these include fatty acids (27), ceramide (28), and even phosphatidic acid, when fully protonated (29). Diacylglycerols also cross phospholipid bilayers with a half-time on the order of 10 msec (30). The aforementioned lipids contain two to five polar oxygen atoms while sterols contain but a single oxygen atom on a large hydrocarbon mass.

ESTIMATION OF CELL SURFACE CHOLESTEROL

That most of the cholesterol in complex eukaryotic cells such as macrophages will exchange with an extracellular lipid sink was recognized long ago as an indication that the plasma membrane was rich in sterols (31). A similar conclusion was drawn early from subcellular fractionation studies (32). Nevertheless, a precise reckoning of the fraction of cholesterol in the plasma membrane has been elusive (33).

Susceptibility to enzyme probes is used widely as a criterion for the exposure or crypticity of proteins, phospholipids, and glycoconjugates at the surface of cells and organelles. In the case of cholesterol oxidase, at least 90% of total unesterified cholesterol was attacked by cholesterol oxidase in glutaraldehyde-fixed red cells (21), fibroblasts (25, 34), Chinese hamster ovary cells (25), and kidney epithelial cells (34); about 80% was oxidized in fixed hepatocytes (25).

While it has been widely observed that the plasma membranes of many cells are enriched in sterols (2), the degree suggested by cholesterol oxidase has been surprising (33). In particular, calculations based on stereological analyses support the conclusion that no more than 24% and 34% of the cholesterol in BHK and MDCK cells, respectively, is in the plasma membrane (33). The reason for the discrepancy is unclear, however the cholesterol oxidase method requires fewer calculations and assumptions. What is needed is a single study in which the two methods are compared. Nevertheless, several lines of evidence have corroborated the premise that almost all of the cholesterol resides in the plasma membrane, at least for fibroblasts. *a*) The cholestenone formed when intact cells were treated with cholesterol oxidase co-migrated precisely with plasma membrane markers in equilibrium sucrose gradient centrifugation (18). *b*) The cholesterol that was not oxidized in intact cells had a distinctive profile on equilibrium sucrose gradients which was clearly different from that of the oxidized cholesterol as well as plasma membrane markers, suggesting that the probe distinguished between two populations of cholesterol residing in separate membranes (18, 20). *c*) When intact cells were treated with cholesterol oxidase, then homogenized and treated with digitonin (a sterol-specific density shift reagent), the profile of the unoxidized cholesterol on equilibrium sucrose density gradients was increased whereas that of the cholestenone product remained unchanged (18). This finding corroborates the supposition that the susceptible and insusceptible sterol pools resided in different membranes. *d*) Newly synthesized [^{3}H]cholesterol initially was not oxidizable in intact cells but became oxidizable after a lag (19). Concomitantly, its distribution on gradients changed to a pattern coinciding with that of the plasma membrane (18). Presumably, the nascent cholesterol inside the cell was not a substrate for the extracellular enzyme but it became oxidizable after it moved to and rapidly flipped across the plasma membrane.

Subcellular fractionation often has suggested that a large fraction of cholesterol resides in the intracellular organelles (2). However, cross-contamination by plasma membrane fragments plagues such density gradient analyses (34). While correction can be made for such contamination through the quantitation of membrane-specific markers, this usually is not done. When contamination is taken into account, however, analytical fractionation corroborates the assignment of > 90% of cholesterol to the plasma membrane of human fibroblasts (34). Finally, physical estimation of the surface area of various cells led to the conclusion that the relative ratios of cell cholesterol to surface area were quite similar in red cell membranes, fibroblasts, and liver cells (34). Since red cells lack internal membranes, these findings support the notion that the major fraction of membrane cholesterol is at the cell surface in the more complex cell types.

The cholesterol oxidase susceptibility of cholesterol, in either chemical or isotopic form, has been used to characterize sterol redistribution in fixed, intact cells after various stimuli and perturbations (20, 35–39). These studies offer broad confirmation of the features just discussed. In no system was less than 80% of the cholesterol in fixed unbroken cells susceptible to the probe. Furthermore, the systematic changes in cholesterol oxidase susceptibility within each internally controlled study support the view that this probe is not indiscriminately oxidizing cholesterol from all parts of the cell but is reporting on the fraction at the cell surface.

ESTIMATING THE SURFACE AREA OF A CELL WITH CHOLESTEROL OXIDASE

Since the cell surface cholesterol can be determined accurately with cholesterol oxidase, the surface area of a given cell can be estimated readily (34). The choles-

terol in the plasma membrane of intact fibroblasts was measured as 44 fmol/cell. From a plasma membrane cholesterol/phospholipid mole ratio of 0.8 and literature values for the molecular cross-sections of phospholipids and cholesterol-phospholipid complexes, a value of 17,500 μm^2 was calculated for the area of the plasma membrane bilayer of the cultured human fibroblast. This result was corroborated by an independent physical method (34).

The large fraction of cell cholesterol oxidized in fixed intact human fibroblasts forces the conclusion that the plasma membrane of these cells contains about 50% of cellular phospholipid. Adding in the contribution of cholesterol suggests that more than half of the total membrane surface area of these cells is attributable to the plasma membrane (34). An entirely different approach, in which plasma membrane was isolated on cationic beads, similarly suggested that approximately 40% of cellular phosphatidylcholine was in the plasma membrane of Chinese hamster ovary cells (40). These values are larger than expected, perhaps because the prototype for subcellular membrane analysis has long been the hepatocyte, a cell particularly rich in intracellular membranes (41). Indeed, the application of cholesterol oxidase to canine hepatocytes suggested that their plasma membranes contribute only about 10% of cellular phospholipid (34).

THE DISPOSITION OF INTRACELLULAR CHOLESTEROL

Staining cells with filipin prior to electron microscopy (42) has made cholesterol visible in plasma membranes and in the organelles on the endocytic pathway: coated vesicles (43), endosomes (44), *trans* Golgi apparatus (45), and lysosomes (43); the other major membrane compartments like the endoplasmic reticulum, mitochondria, and nuclei were not stained (46). The absence of filipin binding, however, could reflect not the lack of cholesterol, but the blocking of the reaction by a membrane constituent, as reported for clathrin in coated vesicles (43). In any case, filipin staining does not afford quantitation.

Cholesterol oxidase offers a remedy to the contamination of organelle isolates by plasma membrane fragments: pretreatment of intact cells to selectively convert surface cholesterol to cholestenone. By eliminating the major source of contamination, this maneuver makes small intracellular sterol pools visible. The distribution on sucrose gradients of the bulk of the unoxidized cholesterol of fibroblast homogenates treated in this way does not coincide with markers for the Golgi apparatus, mitochondria, lysosomes, peroxisomes, nuclei, endoplasmic reticulum or, naturally, plasma membrane (20, 47). Thus, the ~10% of the cholesterol deemed to be intracellular seems not to be generally distributed among the cellular organelles. This lack of a broad distribution is consistent with two premises: *a*) that cholesterol does not diffuse readily among the organelles through the aqueous cytosol (48), and *b*) that cholesterol is apportioned specifically to target membranes (19, 49).

The sucrose density gradient profile of the small amounts of cholesterol mass resistant to cholesterol oxidase roughly coincides with the distribution of endocytic markers in human fibroblasts (20). Furthermore, this intracellular cholesterol pool becomes completely mixed with the cholesterol in the plasma membrane over several hours (20). The clear implication is that the bulk of intracellular cholesterol is endosomal. This is consistent with the high sterol content of purified endosomes and lysosomes (2). (Note that the lipids in these endocytic compartments could arise from ingestion and/or autophagy rather than membrane biogenesis.) The magnitude of the intracellular cholesterol compartment in fibroblasts, ~10% of the total (20), is about that expected from measurement of endocytic plasma membrane (50). In the sense that the endocytic pathway is an intracellular sampling of the cell surface, it appears that the cell takes care to confine cholesterol to a single but complex compartment, the plasma membrane.

STEROL MOVEMENT

Cellular cholesterol is derived from that taken up from the plasma, that liberated from ester stores, and that synthesized in the endoplasmic reticulum (51). The high degree of enrichment of cholesterol in the plasma membrane suggests that the cholesterol from all sources is moved up its gradient and targeted to the cell surface. Several methods have been applied to the study of this process: *1*) plasma membrane isolation on cationic beads (49); *2*) cholesterol oxidase (17, 19, 35–38); *3*) mixing and re-isolation of purified subcellular fractions (52); and *4*) equilibrating the surface of intact cells with an acceptor of plasma membrane cholesterol (53). Rapid transfer of newly synthesized cholesterol to the plasma membrane ($t_{1/2}$ ~10 min) has been demonstrated in Chinese hamster ovary cells using a plasma membrane separation technique (49). Transfer to the cell surface of newly synthesized cholesterol measured by cholesterol oxidase had a half-time of 10 min (54) to 1 h (19) in fibroblasts, and 20-30 min in Leydig tumor cells (36). In ovarian granulosa cells, the half-time of translocation increased from less than 20 min to approximately 2 h in response to gonadotropins (37).

The mechanism by which newly synthesized cholesterol is transferred to the plasma membrane has not been elucidated. Special vesicles may be involved (18, 52); however, the Golgi apparatus does not seem to participate (47, 55).

Several of the intermediates in cholesterol biosynthesis after lanosterol are substrates for cholesterol oxidase (8). When fixed intact fibroblasts were treated with cholesterol oxidase, most of their newly synthesized zymosterol, lathosterol, and 7-dehydro-cholesterol was oxidized (56). Subcellular fractionation confirmed that the bulk of these cholesterol precursors was in the plasma membrane. Since the final steps of cholesterol biosynthesis occur in the endoplasmic reticulum (7) and consume these cell surface pools (56), the late sterol precursors at the cell surface must be reinternalized, a process that can be delineated by cholesterol oxidase (54).

By transforming the large pool of cell surface sterol, cholesterol oxidase has revealed the tiny intracellular pool of newly synthesized cholesterol heading to the plasma membrane. The nascent intracellular cholesterol in human fibroblasts may amount to only ~1% of the cell total (20). Nascent cholesterol did not accumulate in the endoplasmic reticulum after synthesis (18, 47); rather it was quickly moved to more buoyant, cholesterol oxidase-insensitive membranes. These were distinguishable from the plasma membrane and from the bulk of the intracellular cholesterol which is mostly endocytic (20). The resolution of this compartment bearing newly synthesized sterol would not have been feasible without the prior oxidation of the great excess of surface radiosterol.

CONCLUSIONS

The varied applications of cholesterol oxidase described above lead to the following picture of the organization of cholesterol within a test system, the cultured human fibroblast. *a*) The transmembrane movement of cholesterol is fast (characteristic time of less than a few seconds). *b*) About 90% of cell sterol can be assigned to the cell surface. *c*) Almost all of the intracellular cholesterol, approximately 10% of the cell total, is in endocytic membranes, hence directly derived from the plasma membrane. *d*) Newly synthesized sterol, ~1% of the cell total, is associated with special membranes not identified with any of the major organelles (including its source, the endoplasmic reticulum).

The marked nonhomogeneity in cholesterol distribution among the membranes of the cell suggests that cholesterol, whether newly synthesized, released from esters, or delivered by the endocytosis of low density lipoproteins, is continually excluded and/or removed from intracellular membranes and directed to the plasma membrane. The mechanisms by which this specific uphill targeting proceeds remain to be elucidated. One must also wonder what advantage there is to the cell in excluding cholesterol from the cytoplasmic organelles and concentrating it in the plasma membrane.

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