Movement of Zymosterol, a Precursor of Cholesterol, among Three Membranes in Human Fibroblasts*

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Where examined, cholesterol is synthesized in the endoplasmic reticulum; however, its precursor, zymosterol, is found mostly in the plasma membrane. The novel implication of these disparate findings is that zymosterol circulates within the cell. In tracing its movements, we have now established the following: (a) in human fibroblasts, zymosterol is converted to cholesterol solely in the rough ER. (b) Little or no zymosterol or cholesterol accumulates in the rough ER in situ. (c) Newly synthesized zymosterol moves to the plasma membrane without a detectable lag and with a half-time of 9 min, about twice as fast as cholesterol. (d) The pool of radiolabeled zymosterol in the plasma membrane turns over rapidly, faster than does intracellular cholesterol. Thus, plasma membrane zymosterol is not stagnant. (e) [3H]Zymosterol pulsed into intact cells is initially found in the plasma membrane. It is rapidly internalized and is then converted to [3H]cholesterol. Half of the [3H]cholesterol produced returns to the plasma membrane within 30 min of the initial [3H]zymosterol pulse. (f) Nascent zymosterol accumulates in a buoyant sterol-rich intracellular membrane before it reaches the plasma membrane. This membrane also acquires nascent cholesterol, exogenous [3H]zymosterol pulsed into intact cells, and [3H]cholesterol synthesized from the exogenous [3H]zymosterol.

These results suggest that at least one sterol moves rapidly and in both directions among the rough endoplasmic reticulum, a sterol-rich intracellular membrane bearing nascent cholesterol, and the plasma membrane.

The unesterified cholesterol made in cultured human fibroblasts is distributed unequally among at least four compartments. About 90% of the cholesterol mass is found in the plasma membrane (1, 2). Approximately 10% of the cholesterol mass is ascribable to the endocytic membranes derived from the plasma membrane (3). About 1% is found in novel intracellular membranes which are buoyant, enriched in sterols, rapidly labeled by newly synthesized cholesterol, and separable from ER1 and Golgi markers (3-6). A trace of nascent cholesterol is found in the ER,2 its site of synthesis (7).

At least three cholesterol precursors are also highly concentrated in the plasma membrane; these are lanosterol (8), 7-dehydrocholesterol, and zymosterol (9). The mechanisms underlying this nonuniform sterol distribution and the purpose served by it are unclear (7, 10). To better understand membrane lipid topogenesis, we have now analyzed the intracellular movements of zymosterol, the immediate precursor to cholesterol.

EXPERIMENTAL PROCEDURES

Materials—Reagents and isotopes were obtained as described (9). [3H]Zymosterol was synthesized from radiolabeled acetate by triparanol-treated cultured rat hepatoma cells (9). It had a specific activity of ~10^6 dpm/μg. Human foreskin fibroblasts were cultured as before (11).

Lipid Analysis—Reverse phase HPLC of extracted lipids was as described (9). A second HPLC run was used to purify the zymosterol and cholesterol synthesized from radiolabeled acetate.

Conversion of Exogenous Zymosterol—When labeling intact cells, radiolabeled zymosterol was solubilized in 2.5% Triton WR 1339 and added to a final concentration of detergent of <30%. The use of Triton WR-1339 as a vehicle for sterol tracers was patterned after Gaylor and co-workers (12, 13). This agent was benign and did not affect cell growth over at least 1 day. Similar results were invariably obtained when sterol tracers were introduced instead out of an ethanolic solution as described in Ref. 1. For homogenates we used the method described above in which 1 mM NADPH was added as cofactor (9).

Treatment of cells with cholesterol oxidase, homogenization, and equilibrium sucrose gradient analysis were performed as described (3).

RESULTS

Kinetics of Transfer of Nascent Sterols to the Plasma Membrane—Replicate flasks of fibroblasts were incubated with [3H]acetate at 37 °C. At intervals, the intact cells were fixed with glutaraldehyde and treated with cholesterol oxidase (11). The fraction of radioactivity in the oxidized form of the sterols was found to increase in a first-order fashion with a half-time of 9 min for zymosterol and 16 min for cholesterol (Fig. 1).

Approximately 80% of each nascent sterol was accessible to the oxidase in the plateau. Comparable rates and extents of transfer to the cholesterol oxidase-sensitive pool were obtained in similar experiments for two other late sterol intermediates, lathosterol and 7-dehydrocholesterol (data not shown). We interpret these results to signify rapid movement of nascent sterols to the cell surface.

The fraction of radiolabeled zymosterol and cholesterol at the cell surface at steady state approached that of their masses, reported previously (9, 11). The half-time for cholesterol movement to the plasma membrane in Fig. 1 (i.e. ~18
Two pools of cholesterol (see also Ref. 4). The same numbers of C and H atoms, the relative ages of intracellular cholesterol pool was significantly younger than was the membrane zymosterol pool. Indeed, it was almost as young as the intracellular zymosterol pool. While the profiles of the oxidized sterols (Fig. 2) were characteristically broader and denser. This is demonstrated by the juxtaposition of the profiles of the oxidized and unoxidized peaks were better separated in many other experiments than that in Fig. 1. These peaks coincided with those of cholesterol. Fibroblasts were labeled with [3H]acetate for 2 h as described in the legend to Fig. 1. The data were fit by computer to a first-order expression using a nonlinear least squares method.

**Relative Age of the Sterol Pools**—Cells were first incubated with [14C]acetate for 2 h; [3H]acetate was then added for a further incubation of 1 h. The cells were fixed, treated with cholesterol oxidase, and the labeling of sterol pools determined. Because the various sterol species have approximately the same numbers of C and H atoms, the relative ages of different pools can be inferred from the ratios of the two isotopes incorporated into each (4, 9). The data in Table I are interpreted to signify that the cell surface zymosterol pool was only slightly older than its intracellular counterpart, while a greater difference in age was observed for the corresponding two pools of cholesterol (see also Ref. 4). The plasma membrane zymosterol pool was significantly younger than was the intracellular cholesterol pool; indeed, it was almost as young as the intracellular zymosterol pool.

**Subcellular Distribution of Newly Synthesized Zymosterol**—Fibroblasts were incubated for 2 h with [3H]acetate, treated with cholesterol oxidase, homogenized, and centrifuged to equilibrium on sucrose gradients. The profiles of radiolabel in zymosterol, cholesterol, zymostenone, and cholesterol were determined (Fig. 2). [3H]Cholestane and [3H]zymostenone were congruent in buoyant density with a peak at 30% sucrose (d = 1.13 g/cm³; Fig. 2A). This peak coincided with that of cholestane mass, a marker for the plasma membrane (not shown, but see Fig. 6B). The profiles of the unoxidized fractions of the two sterols were coincident in a broad peak with a modal buoyant density of ~35% sucrose (d = 1.16 g/cm³; Fig. 2B). Little or none of the newly synthesized sterols migrated with markers for the smooth or rough ER which are confined to the dense part of the gradient (>40% sucrose; Ref. 5 and Fig. 6B).

While the profiles of the oxidized sterols (Fig. 2A) overlapped those of the unoxidized sterols (Fig. 2B), the latter were characteristically broader and denser. This is demonstrated by the juxtaposition of the profiles of the oxidized and unoxidized form of each sterol in Fig. 2, panels C and D. Furthermore, the oxidized and unoxidized peaks were better separated in many other experiments than that in Fig. 2 (e.g., 3A and 3B).

**TABLE I**

<table>
<thead>
<tr>
<th>Experimnet Sterol</th>
<th>3H</th>
<th>14C</th>
<th>3H/14C</th>
<th>Relative H/3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lanosterol</td>
<td>2,429</td>
<td>1,512</td>
<td>1.61</td>
<td>8.0</td>
</tr>
<tr>
<td>Unoxidized zymosterol</td>
<td>472</td>
<td>693</td>
<td>0.68</td>
<td>3.4</td>
</tr>
<tr>
<td>Oxidized zymosterol</td>
<td>2,172</td>
<td>3,942</td>
<td>0.55</td>
<td>2.8</td>
</tr>
<tr>
<td>Unoxidized cholesterol</td>
<td>1,940</td>
<td>4,175</td>
<td>0.46</td>
<td>2.3</td>
</tr>
<tr>
<td>Oxidized cholesterol</td>
<td>3,141</td>
<td>16,079</td>
<td>0.20</td>
<td>1.0</td>
</tr>
<tr>
<td>Lanosterol</td>
<td>29,180</td>
<td>9,443</td>
<td>3.09</td>
<td>11.4</td>
</tr>
<tr>
<td>Unoxidized zymosterol</td>
<td>2,703</td>
<td>1,452</td>
<td>1.86</td>
<td>6.9</td>
</tr>
<tr>
<td>Oxidized zymosterol</td>
<td>8,364</td>
<td>5,901</td>
<td>1.42</td>
<td>5.3</td>
</tr>
<tr>
<td>Unoxidized cholesterol</td>
<td>8,126</td>
<td>11,078</td>
<td>0.73</td>
<td>2.7</td>
</tr>
<tr>
<td>Oxidized cholesterol</td>
<td>13,726</td>
<td>50,150</td>
<td>0.27</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Fig. 3. The buoyant density profiles of zymosterol arising endogenously and exogenously. Confluent monolayers of fibroblasts were preincubated for 48 h in medium containing 5% lipoprotein-deficient serum. These cells were then incubated with [14C]zymosterol for 1.5 h at 20 °C. The labeled medium was removed, the monolayer rinsed once with buffer, and fresh medium containing 5% lipoprotein-deficient serum plus 0.5 mCi of [3H]acetate was added. After 1 h at 37 °C, the cells were processed as described in the legend to Fig. 2 and gradient fractions analyzed for cholesterol oxidase-insensitive biosynthetic [3H]zymosterol (○) and exogenous [14C]zymosterol (●). Radioactivity is expressed as 10^-3 × dpm.

Additional evidence that the unoxidized zymosterol and cholesterol resided in membranes different from their oxidized counterparts was the selective shift of their buoyant density to higher values with digitonin treatment (4).2

Subcellular Distribution of Internalized Plasma Membrane Zymosterol—Intact fibroblasts were allowed to take up exogenous [14C]zymosterol while also synthesizing [3H]zymosterol from [3H]acetate. The cells were then treated with cholesterol oxidase to eliminate the background of plasma membrane sterols. The buoyant density profiles of the unoxidized exogenous and endogenous zymosterol coincided in a broad and complex peak centered at ~35% sucrose (Fig. 3). This result strongly suggests that zymosterol internalized from the plasma membrane moved to the same intracellular locus as newly synthesized zymosterol.

Metabolism of Plasma Membrane [3H]Zymosterol—Intact cells were loaded with exogenous [3H]zymosterol at 20 °C. At this temperature, the probe was entirely accessible to cholesterol oxidase over 1 h in intact cells; we infer that it remained in the plasma membrane. If, instead, the cells were shifted to 37 °C for 1 h prior to treatment with cholesterol oxidase, ~10% of the label accumulated in the cell interior, some of which was converted to [3H]cholesterol. That is, ~10% of the total cellular radioactivity was recovered in zymosterol + cholesterol + cholestenone and ~90% in zymostenone. This small degree of internalization matches the steady state distribution of endogenous zymosterol shown in Fig. 1 and described in Ref. 9.

The internalized [3H]zymosterol was progressively converted to cholesterol despite competition from its unlabelled endogenous counterpart (Fig. 4A). While this radiocholesterol was mostly inaccessible to cholesterol oxidase at the outset, the fraction which could be oxidized in intact cells increased during the incubation at 37 °C (Fig. 4B). We interpret these data to signify that some of the exogenous [3H]zymosterol pulsed into the plasma membrane entered the cell and moved to the locus of cholesterol biosynthesis where it was converted to [3H]cholesterol. This nascent [3H]cholesterol then moved to the plasma membrane where it was oxidized with kinetics similar to that of endogenous cholesterol (Fig. 1).

Subcellular Distribution of Sterols Derived from Internalized

Fig. 4. Conversion of exogenous [3H]zymosterol to [3H]cholesterol and its return to the plasma membrane. Replicate flasks of fibroblasts were labeled with [3H]zymosterol as described in the legend to Fig. 3, washed, and fresh unlabeled medium was added. The flasks were then incubated at 37 °C for the time indicated. Each flask then was chilled and rinsed with ice-cold buffer; the cells were dissociated with trypsin, washed, fixed on ice, and treated with cholesterol oxidase. The lipids were extracted and the radioactivity in sterols determined by HPLC. A, the time course of synthesis of [3H]cholesterol from [3H]zymosterol. Because multiple culture flasks were used, each point was normalized as 100 × ([3H]cholesterol + [3H]cholestenone)/(3H)cholesterol + [3H]cholestenone + [3H]zymosterol). B, the time course of the fraction of [3H]cholesterol in a cholesterol oxidase-susceptible form. The data are expressed as 100 × ([3H]cholestenone)/(3H)cholestenone + [3H]cholesterol).

Plasma Membrane [3H]Zymosterol—Cells were labeled with exogenous [3H]zymosterol at 20 °C for 1 h, then incubated for 1 h at 37 °C to allow partial internalization and conversion of the [3H]zymosterol. The cells then were treated with cholesterol oxidase, homogenized, and centrifuged to equilibrium on a sucrose gradient. The profiles of [3H]zymosterol and [3H]cholesterol in the oxidized and unoxidized forms were determined.

In the experiment shown in Fig. 5, 12% of the label associated with the cells was found to be resistant to cholesterol oxidase. As in Fig. 3, the unoxidized [3H]zymosterol was distributed in a broad and complex peak between 30 and 40% sucrose (Fig. 5A, open circles). It was clearly distinguishable from the [3H]zymostenone profile (Fig. 5A, filled circles). Approximately 30% of the internalized label was recovered in radiocholesterol. Of this, 68% was susceptible to cholesterol oxidase. The buoyant density profiles of the oxidized and unoxidized [3H]cholesterol pools differed (Fig. 5, panel B). The distribution of the oxidized [3H]cholesterol was very similar to that of oxidized [3H]zymosterol (Fig. 5, panel C) as well as to cholestenone mass, a marker for the plasma membrane (not shown, but see Fig. 6B and Ref. 4). The profile of unoxidized [3H]cholesterol was virtually superimposable on that of the unoxidized [3H]zymosterol (Fig. 5, panel D). None

Bidirectional Sterol Movement in Fibroblasts

Fig. 5. Buoyant density distribution of [3H]sterols made from exogenous [3H]zymosterol by intact cells. Fibroblasts were labeled with [3H]zymosterol as described in the legend to Fig. 3, the monolayer rinsed once, and fresh unlabeled medium pre-equilibrated at 37 °C added to the flask. After 1 h at 37 °C, the medium was removed; the cells were dispersed, washed, treated with cholesterol oxidase, homogenized, and analyzed as described in the legend to Fig. 2. A, distribution of [3H]zymostenone (●) and [3H]zymosterol (○). B, distribution of [3H]cholestenone (■) and [3H]cholesterol (□). C, data from A and B were replotted to show the similarity in the distribution of [3H]zymostenone (●) and [3H]cholestenone (■). D, data from A and B were replotted to show the similarity in the distribution of [3H]zymosterol (○) and [3H]cholesterol (□). Radioactivity is expressed as counts/min.

of the label could be assigned to the rough ER, which equilibrated at >247% sucrose (see below).

Site of Conversion of Zymosterol to Cholesterol—Fibroblast homogenates were spun to equilibrium on sucrose gradients and each gradient fraction was tested for its ability to convert [14C]zymosterol to [14C]cholesterol. This activity equilibrated at a buoyant density greater than 47% sucrose (d ≥ 1.20 g/cm3; Fig. 6A). The [14C]cholesterol product was recovered in these dense membranes upon recentrifugation.

[3H]Desmosterol was also tested as a substrate, although this precursor does not seem to be a natural intermediate in cholesterol biosynthesis in human fibroblasts (9). The activity catalyzing the conversion of desmosterol to cholesterol coincided with that for the conversion of zymosterol to cholesterol (Fig. 6A).

Cholesterol mass appeared as a single peak centered at a buoyant density of 31% sucrose (d = 1.13 g/cm3; Fig. 6B). This was taken as a marker for the plasma membrane (4). The major peak of RNA, a marker for rough ER, equilibrated at the same density as the cholesterol-synthesizing activities (Fig. 6D). A similar high buoyant density for rough ER is found in liver (12). We also showed that nuclei, the bulk of which were removed by the routine low speed recentrifugation of the homogenate, lacked the sterol converting activities.

DISCUSSION

These data support the following hypothetical scheme for the movements of zymosterol and cholesterol within the human fibroblast.

(a) Cholesterol is synthesized in the rough ER (Fig. 6). The same is likely to be true of zymosterol (12).

(b) It appears that newly synthesized zymosterol and cholesterol are so efficiently transferred that no more than a trace of either is detected in the region of smooth ER (~42% sucrose; Ref. 5) or rough ER (≥50% sucrose; Fig. 6 and Ref. 5) following subcellular fractionation (Figs. 2, 3, and 5).

(c) Nascent zymosterol and cholesterol enter dynamic intracellular pools (Table 1). These pools are located in the same broad and complex membrane peak. It has a modal density ~1.16 g/ml (Figs. 2 and 3) and does not correspond to any of the major organelles (3, 5). The profiles of these nascent sterols are strongly shifted in density by digitonin, a sterol-seeking lectin glycoside, while markers for ER (17) and Golgi apparatus (5) show little or no density shift. Since the degree of density shift by digitonin reflects the sterol content of the membranes (18), we refer to the membranes bearing nascent zymosterol and cholesterol as a sterol-rich organelle.

(d) Zymosterol appears at the plasma membrane without a lag and with a half-time of ~9 min, invariably faster than cholesterol (Fig. 1). These rates are much faster than those observed for the non-mediated transfer of cholesterol between membranes (19). The absence of a lag in the time course of fractional oxidation suggests that these sterols are not obliged to traverse a series of compartments on their way from ER to plasma membrane, as is generally the case for even the most rapidly transported cell surface and secretory proteins (20). The first-order kinetics of fractional accessibility seen in Fig. 1 may therefore reflect the random (age independent) sampling or turnover of the pool in the intracellular sterol-rich organelle.

(e) Plasma membrane zymosterol returns to the cytoplasm. This is shown by the evidence that the plasma membrane pool of zymosterol is turning over faster than the intracellular
pool of cholesterol and may be near equilibrium with its cytoplasmic counterpart (Table I). Furthermore, zymosterol introduced from without into the plasma membrane becomes progressively resistant to cholesterol oxidase and is converted to cholesterol (Fig. 4).

(f) All of the zymosterol which returns to the cytoplasm from the plasma membrane is found in the same membranes that bear the newly synthesized sterols: the sterol-rich organelle (Fig. 3 and 5). In contrast, most of the cholesterol internalized from the plasma membrane does not congregate in the sterol-rich organelle but in endocytic membranes (3). This pattern constitutes the major difference detected thus far between the behavior of cellular zymosterol and cholesterol.

(g) Zymosterol internalized from the plasma membrane returns to the rough ER. This conclusion is supported by two observations. First, the rough ER is the exclusive site for the conversion of zymosterol to cholesterol (Fig. 6). Second, biosynthetically labeled zymosterol can be completely chased into cholesterol during an overnight incubation. We do not know what proportion of the cholesterol synthesized at any moment is derived from zymosterol returning from the plasma membrane or the sterol-rich organelle, as opposed to that newly arising within the ER. The prompt biosynthetic labeling of cholesterol from $^{3}H$acetate (9, 11) might mean that the ER utilizes a slip-stream of zymosterol arising in situ in preference to the relatively large pool of unlabeled zymosterol in the plasma membrane.

(h) Given this evidence that zymosterol movement is bidirectional, the rapid appearance of the nascent sterol in the sterol-rich organelle and the plasma membrane may signify some degree of isotope exchange rather than net transfer. If so, some molecules of zymosterol could pass through part or all of this circuit more than once before their final conversion to cholesterol.

The purpose of the premature transport of late sterol precursors out of the ER is unknown. They might simply be indiscriminately transferred along with cholesterol to the plasma membrane. Alternatively, the cell might benefit from using the plasma membrane as a large capacity reservoir for these biosynthetic intermediates. Finally, a regulatory role might be served by bringing cholesterol precursors through the plasma membrane compartment. In any case, there is no evidence and no reason to postulate that zymosterol molecules are obliged to go to the cell surface before conversion to cholesterol; nevertheless, this possibility warrants investigation.

The pathway through which sterols are taken to and from the plasma membrane is obscure. One type of bidirectional pathway could involve a specific sequence of reversible transport steps, such as rough ER $\rightarrow$ sterol-rich organelle $\rightarrow$ plasma membrane. In this hypothetical scheme, the sterol-rich organelle is an intermediary between the ER and plasma membrane. Alternatively, there could be a cycle of unidirectional steps between the ER and plasma membrane or simply random mingling of sterol molecules among membranes.

One mechanism mediating bidirectional flow is simply the diffusion of sterol molecules between membranes through the aqueous phase. There is evidence both for and against this idea (19, 21). In the present context, this model leaves unexplained both the high rate of sterol movement (Fig. 1) and the striking inhomogeneity in the distribution of cellular sterols (1–3). An attractive alternative is that cytoplasmic proteins move sterols, either through the aqueous diffusion of stoichiometric complexes or by facilitating sterol transfer during membrane collision (22). Membrane fission and fusion could convey sterols in both directions without their ever exiting the bilayer.

Might sterols be carried by membrane flow from ER to Golgi plasma membrane as is the rule for cell surface proteins? The participation of the Golgi apparatus in nascent sterol delivery was not supported by a recent study with brefeldin A (23), nor do nascent sterols accumulate in the Golgi (5). Furthermore, newly synthesized glycoproteins and phospholipids reach the plasma membrane with different kinetics from that of cholesterol (24). The return phase is also problematical: while a trickle of plasma membrane glycoproteins and glycolipids returns to the Golgi apparatus via endocytosis (cf. Ref. 24), these compounds are not known to reach the ER (20). Thus, the vehicle which returns plasma membrane zymosterol to the ER may well not be the endocytic/biosynthetic endomembrane system.

That sterols regularly circulate between the plasma membrane and the ER has not been directly demonstrated previously, although evidence for this process is the synthesis of esters (25) and steroids (16) from cell surface cholesterol. It may be characteristic of the system that the return of cell surface cholesterol to the cytoplasm is constrained compared to that of its precursors. Nevertheless, these sterols may share common pathways. The mechanism by which they are concentrated in the plasma membrane despite their mobility remains obscure.

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REFERENCES