Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol

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Abstract The abundance of cell cholesterol is governed by multiple regulatory proteins in the endoplasmic reticulum (ER) which, in turn, are under the control of the cholesterol in that organelle. But how does ER cholesterol reflect cell (mostly plasma membrane) cholesterol? We have systematically quantitated this relationship for the first time. We found that ER cholesterol in resting human fibroblasts comprised ~0.5% of the cell total. The ER pool rose by more than 10-fold in less than 1 h as cell cholesterol was increased by ~50% from below to above its physiological value. The curve describing the dependence of ER on plasma membrane cholesterol had a J shape. Its vertex was at the ambient level of cell cholesterol and thus could correspond to a threshold. A variety of class 2 amphiphiles (e.g., U18666A) rapidly reduced ER cholesterol but caused only minor alterations in the J-curve. In contrast, brief exposure of cells to the oxysterol, 25-hydroxycholesterol, elevated and linearized the J-curve, increasing ER cholesterol at all values of cell cholesterol. This finding can explain the rapid action of oxysterols on cholesterol homeostasis. Other functions have also been observed to depend acutely on the level of plasma membrane cholesterol near its physiological level, perhaps reflecting a cholesterol-dependent structural or organizational transition in the bilayer. Such a physical transition could serve as a set-point above which excess plasma membrane cholesterol is transported to the ER where it would signal regulatory proteins to down-regulate its further accumulation.—Lange, Y., J. Ye, M. Rigney, and T. L. Steck. **Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol.** ***J. Lipid Res.*** **1999.** 40: **2264–2270.**

Supplementary key words homeostasis • sensor • 25-hydroxycholesterol • U18666A

Sterols such as cholesterol are ubiquitous in eukaryotic cells. Cholesterol homeostasis is under elaborate feedback control, but it is not clear how cells define their need for it, sense its abundance, and signal the difference to effectors of its biosynthesis, ingestion, export and storage as ester droplets (1–3). How, in particular, is the bulk of cell cholesterol (mostly in the plasma membrane) perceived by the multiple control proteins residing in the ER? Some of these proteins have "sterol-sensing domains" (4, 5) which could read the level of cholesterol as a homeostatic cue (1). By these means, the pool of cholesterol in the ER might regulate the activity of all the ER control proteins coordinately (6). In that case, the question becomes: how does the small cholesterol pool in the ER sense the sufficiency of the bulk pool?

The abundance of plasma membrane cholesterol could set the magnitude of the ER pool if the former circulated through the latter in a regulated fashion (6, 7). That is, a brisk throughput of plasma membrane cholesterol might flush out the ER pool and continuously adjust it to the appropriate level. In this scheme, incoming cholesterol might be governed by a sensor which allocates to the ER a fraction of plasma membrane cholesterol in excess of a threshold set-point (6, 8). In the simplest case, the size of the ER pool would be defined relative to that in the plasma membrane by the rate constants for cholesterol flux between the two pools.

We have tested this model by measuring how the ER cholesterol pool responds to variations in bulk (plasma membrane) cholesterol; whether this relationship in fact shows a threshold; and how this relationship is affected by agents known to alter cholesterol homeostasis. Our results suggest a simple mechanism by which the plasma membrane could set this threshold and sense its cholesterol.

MATERIALS AND METHODS

Materials

[Oleoyl-1-^{14}C]coenzyme A was purchased from DuPont NEN; [1α,2α(n)-^{3}H]cholesteryl oleate was from Amersham Life Science, Inc. U18666A was generously provided by Pharmacia and Upjohn Co. (Kalamazoo, MI). 25-Hydroxycholesterol was purchased from Sigma. A 20% (w/v) solution of hydroxypropyl-β-cyclodextrin (HPCD) from Research Plus, Inc. (Bayonne, NJ)

Abbreviations: ER, endoplasmic reticulum; HPCD, hydroxypropyl-β-cyclodextrin; LPDS, lipoprotein-deficient serum.

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was complexed with cholesterol to ~0.1 mol/mol, as described (9). Human foreskin fibroblasts and rat FU5AH hepatoma cells were cultured as described (10, 11) in Dulbecco's minimum essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and either 10% fetal bovine serum or 5% lipoprotein-deficient serum (LPDS), prepared from fetal bovine serum (12).

Quantitation of ER cholesterol

As a measure of the ER pool, we determined the cholesterol esterified when homogenates were reacted to completion with [^{14}C]oleoyl-CoA (13). Briefly, cells were homogenized and cleared of intact cells and debris by low speed centrifugation. Dithiothreitol (1 mm) and bovine serum albumin (1 mg/ml) were added to aliquots of the supernatant. Thirty μm [^{14}C]oleoyl-CoA was added and the reaction was allowed to proceed for 2 h at 37°C. After the addition of a [^{3}H]cholesteryl oleate standard to correct for subsequent losses, cholesteryl esters were isolated by thin-layer chromatography and their radioactivity was determined. The mass of cholesterol esterified was calculated from the incorporated ^{14}C radioactivity, using the specific activity of the added [^{14}C]oleoyl CoA. Values were expressed relative to cell protein (14) or total cell cholesterol, determined by HPLC (10, 15). Duplicate aliquots were analyzed; their values agreed within 6%. Note that this mode of analysis is not affected by the level of ACAT activity which we have shown to be sufficient to esterify the entire ER cholesterol pool during the 2-h incubation under all conditions tested here (13). For example, while it stimulates acyl-CoA:cholesterol acyltransferase activity (16, 17), the addition of 25-hydroxycholesterol to homogenates did not significantly alter the run-off values for the ER cholesterol pool (not shown).

Determination of ER cholesterol as a function of bulk cell cholesterol

Cell monolayers were switched to medium containing 5% LPDS plus 1–5% HPCD (to deplete plasma membrane cholesterol) or 10% serum plus 1–3% HPCD complexed with cholesterol (to increase plasma membrane cholesterol) (9, 18). After a 15–20-min incubation at 37°C, the medium was removed, the cells were rinsed, and medium containing 5% LPDS or 10% serum, respectively, was added for a 45–60 min incubation at 37°C to allow the ER pool to respond. Finally, the cells were dissociated, homogenized, and cell and ER cholesterol as well as cell protein were determined. To combine data from multiple experiments, we expressed the results as relative ER cholesterol: that is, we divided the value for the ER cholesterol by that for the whole cell protein in each flask and divided the quotients for treated samples by the quotient for the unperturbed control.

RESULTS

Kinetics of response of the ER pool to plasma membrane cholesterol

We used the complexing agent, HPCD, to rapidly extract a portion of plasma membrane cholesterol from intact cells. The response of the ER pool was then determined (**Fig. 1**). A 15-min exposure to HPCD, which reduced cell cholesterol by ~25%, led to an immediate drop in ER cholesterol (half-time of 12 min) to a plateau value of 26% of the control. The decrease in ER cholesterol was apparently a physiological response to the extraction rather than a direct consequence of it, as most of the change occurred after the HPCD was removed.

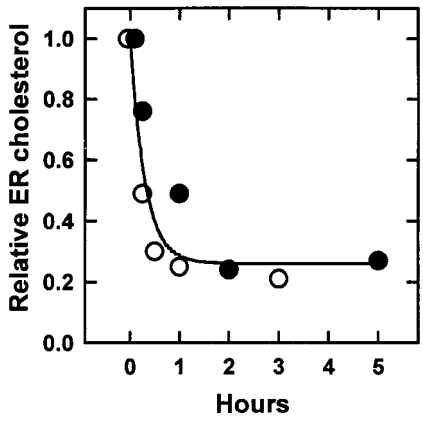

Fig. 1. Response of ER cholesterol to cell cholesterol depletion. In each of the two separate experiments shown, five replicate flasks of fibroblasts were switched to medium containing 5% LPDS ± 2% HPCD at time zero. After incubation for 15 min at 37°C, one flask lacking HPCD (taken as the zero time point) and one with HPCD (taken as the 15 min point) were processed. Medium containing 5% LPDS was added to the remaining 3 flasks. After incubation for the times indicated, ER and cell cholesterol and cell protein were determined (13). A first order curve is shown. At the end of the experiment, the average control and depleted cells had, respectively, 40.8 and 30.4 μg cholesterol/mg protein of which the control and depleted ER comprised 0.45 and 0.11 nmol cholesterol/mg protein (or 0.43 and 0.14 % of total cholesterol).

Dependence of ER cholesterol on plasma membrane cholesterol

Plasma membrane cholesterol was varied below and above its resting level by briefly exposing flasks of fibroblasts to HPCD and complexes of HPCD with cholesterol, respectively. The extraction of plasma membrane cholesterol caused a reduction of ER cholesterol, as in Fig. 1, while supplementation with HPCD-cholesterol complexes evoked a rise in ER cholesterol (**Fig. 2A**). The fact that adding a small amount of cholesterol to the HPCD (~0.1 mol/mol) countered its ability to lower ER cholesterol is evidence that its action was cholesterol-specific.

The variation of ER cholesterol with total cell cholesterol described a J-shaped curve (Fig. 2A). That is, the positive response of the ER pool to increments in plasma membrane cholesterol increased abruptly at a turning point. Above this vertex, ER cholesterol typically doubled with each 10% increment in cell cholesterol. Below the vertex, the ER cholesterol fell to approximately one-third of its resting value as cell cholesterol was reduced by 30% (Fig. 2A, inset). Overall, the ER cholesterol pool changed by more than 10-fold as cell cholesterol was varied by approximately 50%. Notably, the vertex of the J-curve in every experiment was close to the values for ambient cholesterol in the untreated controls (▲ in Fig. 2A).

As a control, we determined the rate of cholesterol esterification in intact cells as a function of plasma membrane cholesterol (**Fig. 3**). Clearly, this relationship closely paralleled that for the ER cholesterol pool size determined in vitro (Fig. 2A). That is, the curve was J-shaped and its vertex corresponded to the resting level of cell cholesterol. In the

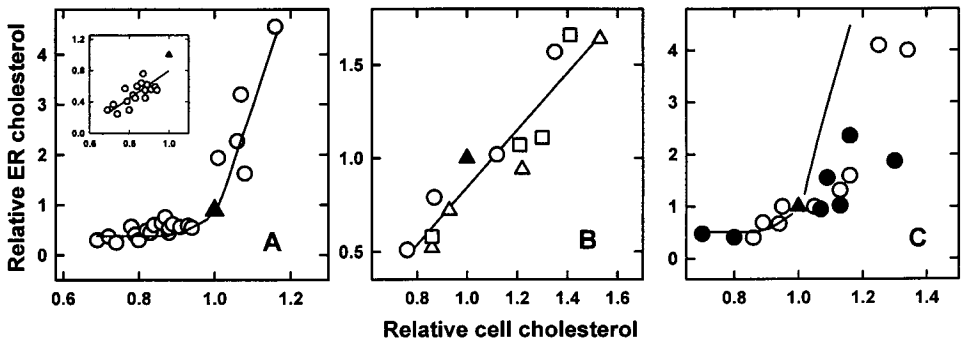

Fig. 2. Dependence of ER cholesterol on plasma membrane cholesterol. (A) Unperturbed cells. In each of ten separate experiments, the plasma membrane cholesterol in replicate flasks of fibroblasts was decreased or increased with various amounts of HPCD ± cholesterol and, after a further incubation of 45–60 min, ER cholesterol, cell cholesterol and cell protein were determined. Values were normalized to the controls not treated with HPCD (▲). The averages of the control values were 0.47 ± 0.2 (SD) nmol ER cholesterol/mg cell protein and 40 ± 4.9 (SD) μg cell cholesterol/mg cell protein. Hence, ER cholesterol was 0.45% of total cell cholesterol. Inset: plot of the values for cholesterol-depleted cells, fit to a straight line. (B) Effect of an oxysterol. As in panel (A), except that, in these three experiments, flasks were pre-incubated for 3 h at 37°C in growth medium containing 25 μm 25-hydroxycholesterol. As the oxysterol and its esters are removed chromatographically, they do not contribute to the values obtained. The averages for controls not treated with HPCD (▲) were 1.3 ± 0.2 nmol ER cholesterol/mg cell protein and 32.6 ± 1.1 μg cell cholesterol/mg cell protein; hence, ER cholesterol was 1.5% of total cell cholesterol. The solid line shows a linear least mean squares fit to the data. (C) Effect of a class 2 amphiphile. As in panel (A), except that the flasks were pre-incubated at 37°C for 4 h (●, two experiments) or 17 h (○, three experiments) in growth medium containing 2.5 μm U18666A. Average ER cholesterol in controls untreated with HPCD (▲) was 0.58 and 0.34 nmol/mg protein and 0.59% and 0.27% of total cell cholesterol at 4 h and 17 h, respectively. The solid line in panel C is the freehand fit to the data for the untreated cells in Fig. 2A; see text.

steep part of the curve, the rate of esterification in vivo rose 3-fold, on average, as cell cholesterol was supplemented by 10%.

Effect of oxysterols on ER cholesterol

After a brief pretreatment of cells with 25-hydroxycholesterol, the dependence of ER cholesterol on plasma membrane cholesterol was no longer J-shaped (Fig. 2B). Rather, the responsiveness of the treated cells to increments in plasma membrane cholesterol was flattened to about the same slope as in the limb of the curve for normal cells in Fig. 2A below its vertex. Furthermore, the ER cholesterol pool was elevated at all plasma membrane cholesterol values; e.g., it was increased by ~3-fold in the resting cells (▲) in Fig. 2B. As expected (see Discussion), the oxysterol treatment caused the total cell cholesterol to fall by ~20% (see legend to Fig. 2A and 2B).

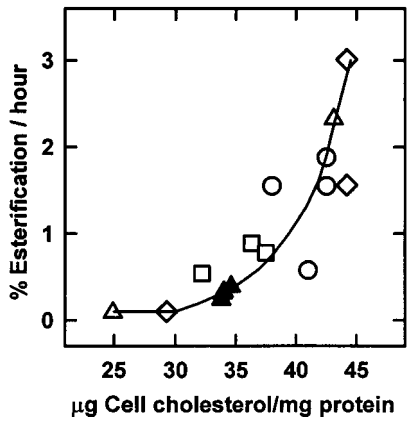

Fig. 3. Dependence of the esterification of plasma membrane [^{3}H]cholesterol in vivo on plasma membrane cholesterol. In each of the three separate experiments shown, fibroblast cultures were pulse-labeled with [^{3}H]cholesterol for 15 min at room temperature and briefly incubated with HPCD ± cholesterol as in Fig. 2. The monolayers were then incubated for 2.5 h at 37°C in medium containing 5% LPDS and extracted to determine the rate of esterification of [^{3}H]cholesterol (13). The points for the three untreated controls are designated as ▲. The solid line gives a freehand fit to the data.

Effects of amphiphiles on ER cholesterol

A wide variety of pharmacological and experimental agents, called class 2 amphiphiles, cause cells to behave as if they sensed a deficiency in their cholesterol (see Discussion and ref. 7). This behavior is illustrated in **Fig. 4**, where two such agents, the hydrophobic amines U18666A and imipramine, are seen to elicit an immediate and steady rise in total cell free cholesterol. Cell cholesterol increased with respect to cell protein at ~2.5%/h. This rate is substantial considering that exponentially growing cells which double in ~20 h would normally accumulate cholesterol at ~3.5%/h overall.

Figure 5 shows that the sustained rise in cell cholesterol in cells treated with class 2 amphiphiles is accompanied by a reduced level of ER cholesterol. When fibroblasts were exposed to U18666A, their ER pool fell to about 40% with a half-time of less than 2 h (panel A). In hepatoma cells (panel B), the response was complete within 20 min, too fast to determine a reliable time constant. Several other

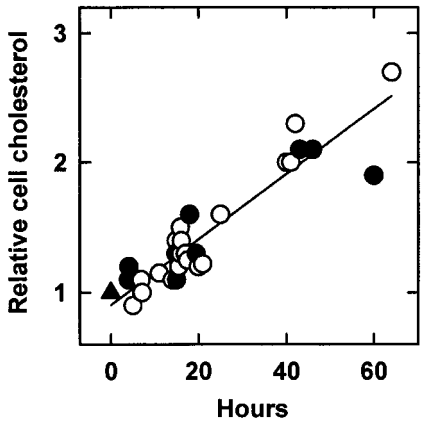

Fig. 4. Effect of U18666A and imipramine on cell cholesterol content. Fibroblasts were incubated at 37°C in growth medium plus 2.5–5.0 μm U18666A (●) or 80 μm imipramine (○) for the times indicated. The cells then were suspended, washed, and assayed for total cholesterol and protein mass. The least mean squares fit is shown.

TABLE 1. Effect of amphiphiles on ER cholesterol

Agent	Concentration	Relative ER cholesterol
	μM	
None	—	1.00
Monensin	2	0.58
Nigericin	3	0.53
U18666A	5	0.42
Trifluoperazine	20	0.41
Progesterone	30	0.54
Lysophosphatidylcholine	40	0.68
Imipramine	80	0.58
Chloroquine	80	0.39
Veratramine	5	0.76
Jervine	9	0.73
Cyclopamine	12	0.76
Tomatidine	50	0.52

In several experiments, replicate flasks of hepatoma cells were incubated for 1.5–2.0 h in growth medium containing the indicated agents. The ER pool was then analyzed as described in Fig. 2. Values are the means of duplicate determinations which agreed to within 5%.

amphiphiles of various types caused a similar reduction of ER cholesterol (**Table 1**). The first set of eight agents are among those originally designated class 2 (7). The second set of four compounds are teratogens capable of inducing holoprosencephaly; our results are consistent with the recent suggestion that these plant steroidal amines behave as class 2 agents (19).

The dependence of ER cholesterol on total cell cholesterol in fibroblasts incubated with U18666A for either 4 h or 17 h was found to describe a J-shaped curve (Fig. 2C) similar to that seen in untreated fibroblasts (Fig. 2A). However, more cell cholesterol was required to increase the ER cholesterol pool in treated cells than in normal cells; this can be appreciated by comparing Fig. 2C with Fig. 2A (the curve for which is superimposed on Fig. 2C). We attribute this effect to the accumulation of cell cholesterol in the endocytic spaces of these cells (see Discussion).

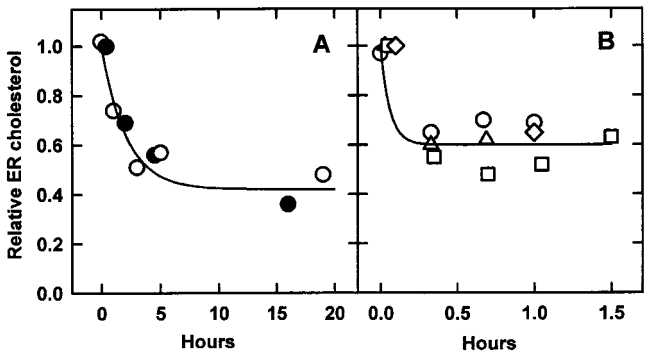

Fig. 5. Effect of U18666A on ER cholesterol. In each of several separate experiments, fibroblasts (panel A) and hepatoma cells (panel B) were incubated at 37°C in growth medium containing 2.5 μm U18666A for the times indicated and ER cholesterol, cell cholesterol and cell protein were determined. Average ER cholesterol in controls not treated with U18666A (zero time points using 0.2% ethanol as a solvent control) were 0.65 and 2.2 nmol/mg protein or 0.59% and 3.6% of total cell cholesterol for fibroblasts and hepatoma cells, respectively. The kinetic curves were fit to first order expressions with half times of 1.45 h (panel A) and 2.5 min (panel B) and equilibrium values of 0.42 and 0.60, respectively.

DISCUSSION

We have defined quantitatively a relationship between cholesterol in the plasma membrane and the ER of human fibroblasts. The ER pool was taken as the total cholesterol esterified when homogenates were reacted to the limit in an in vitro run-off assay. In the original evaluation of the method (13), this pool was shown to be small (normally <1% of total fibroblast cholesterol) and to vary widely (~20-fold) and functionally (i.e., in the homeostatic direction) in response to various treatments of the intact cells (oxysterols, cholesterol oxidase, sphingomyelinase and feeding or withholding low density lipoproteins). The results obtained in the present study further validate the assay. They show, for example, a close parallel between the size of the pool in question (Fig. 2A) and the rate of cholesterol esterification in vivo (Fig. 3) which would appear to reflect cholesterol availability in the ER (20). The in vitro run-off assay is superior to the rate of esterification in vivo as a measure of ER cholesterol pool size because the latter may be responsive to other variables (21) such as stimulation by endogenous oxysterols (15). Moreover, in vivo esterification does not give quantitative values for ER cholesterol as does the run-off assay.

Using extracellular HPCD to manipulate cell surface cholesterol selectively (9, 17, 22), we showed that fibroblasts rapidly translate modest excursions of plasma membrane cholesterol into large changes in the ER pool. These findings are consistent with a negative feedback system in which small increments in plasma membrane cholesterol above a prescribed threshold increase the transfer of cholesterol to the ER, prompting the various control proteins therein to reduce cellular free cholesterol. Positive

feedback by regulatory oxysterols made from incoming plasma membrane cholesterol in the ER and mitochondria might sharpen such responses (6). Furthermore, the J-curve of a given cell might be even more acute than seen in Fig. 2A, given that hyper-sharp step functions in individual cells would be obscured by heterogeneity in the population. In addition, physiological response to increased plasma membrane cholesterol may be even sharper than that seen in these J-curves, as only about half of the increment in cell cholesterol is in the plasma membrane (Y. Lange and J. Ye, unpublished results). On the other hand, the dependence of ER on plasma membrane cholesterol need not be sharply inflected to achieve tight regulation, as small fluctuations in the ER cholesterol pool might elicit high-order responses from the control proteins embedded therein, as has been suggested for acyl-CoA: cholesterol acyltransferase (16, 17).

Physiological, pharmacological and genetic influences might alter the shape and/or setting of the dependence of ER cholesterol on the plasma membrane pool. This appears to be the case for certain oxysterols (Fig. 2B). These effectors send signals of cholesterol excess to regulatory elements in the cell; i.e., they stimulate cholesterol esterification and inhibit sterol biosynthesis and the expression of low density lipoprotein receptors (7, 16, 23). Some effects of oxysterols are mediated by gene expression and occur on a time scale of several hours (24, 25). However, compounds such as 25-hydroxycholesterol can act within minutes (7, 26). The rapidly acting oxysterols could bind directly to control proteins in the ER (3, 4, 16, 17). Alternatively, Fig. 2B supports the hypothesis that all of the various short-term effects of this class of oxysterols are mediated by a rapid expansion of the ER cholesterol pool, which then modulates the proteins embedded in those membranes (13). It is clear that these oxysterols do not shift the position of the J-curve; it is possible that they override the plasma membrane cholesterol-sensing mechanism that normally governs the ER pool.

Class 2 amphiphiles caused free cellular cholesterol to accumulate without limit (Fig. 4), presumably through a decrease in its esterification and an increase in its biosynthesis and ingestion via low density lipoproteins receptors (6, 27). Figure 5 and Table 1 suggest that this effect is mediated by a reduction of ER cholesterol. The reduced ER pool might be a common outcome of the various known actions of this broad class of drugs at diverse and unrelated sites. Alternatively, all of these agents might interact with a single target. Four such candidates have recently been discussed: the multidrug resistance P-glycoprotein (28), the MTP multidrug resistance protein (29), the NPC1 protein (5), and an unusual anionic phospholipid, called bis(monoacylglycero)phosphate or lysobisphosphatidic acid (30). It is notable that the last three of these are located in late endosomes, lysosomes, and/or lamellar bodies (29–33), as these are the compartments in which the excess cholesterol accumulates in amphiphile-treated cells (11, 27, 30, 34, 35). A block in the export of cholesterol from multivesicular late endosomes could lead to their conversion to lamellar bodies, a related membrane lipid-filled buoyant vacuole (36). Mature lysosomes also appear to be converted to lamellar bodies under these conditions (11). The source of the accumulated cholesterol appears to be ingested low density lipoproteins (30, 37) and/or endocytosed plasma membrane bilayer (11).

It therefore seems plausible that the observed reduction in ER cholesterol in drug-treated cells results from a sequestration of cell cholesterol in their endocytic compartments. It has been suggested that the amphiphiles interfere with transport from endocytic spaces to the ER (37, 38). Alternatively, the action of these drugs could be to divert plasma membrane cholesterol to endocytic spaces; the deficiency in the plasma membrane compartment would then be signalled to the ER pool through the regulated circulation described at the beginning of this article and demonstrated in Fig. 2. [This premise is supported by the increased resistance of amphiphile-treated cells to amphotericin B (38), a sterol-specific lytic agent (37).] The consequent reduction in ER cholesterol would prompt the homeostatic elements therein to replace the plasma membrane cholesterol displaced to the endocytic compartments. In this scenario, the ER would function physiologically in the drug-treated cells, consistent with the nearly normal functional relationship between their plasma membrane and ER pools (Fig. 2C). These data also suggest that the cholesterol sensor which cues the ER does not respond to either the endocytic compartment or to total cell cholesterol, as these rise (Fig. 4 and refs. 11, 30) as ER cholesterol falls (Fig. 5).

Finally, our data suggest a mechanism by which the plasma membrane might set its threshold for cholesterol, sense the ambient cholesterol level, and represent the difference to the ER. We start with the premise that the acute responsiveness of the ER pool (Fig. 2A) manifests a high-order cholesterol-dependent signaling mechanism. This could be a cholesterol-sensing protein or a cholesterol-dependent bilayer lipid structure, phase or domain (39). In the latter case, excess plasma membrane cholesterol might be sent to the ER in proportion to the magnitude of the induced cholesterol-dependent structure. Increased ER cholesterol would elicit manifold feedback responses (see Introduction). That the putative physical transition serves as a homeostatic set-point is supported by the finding that the vertex of the J-curve coincides with the ambient level of cholesterol set by the cell (▲ in Fig. 2A). According to this hypothesis, the sufficiency of cholesterol would not be pegged to cell protein or lipid or to the magnitude of the plasma membrane bilayer but rather to the initiation of the bilayer transition.

Earlier evidence for acute cholesterol-dependent changes in the physical state of the bilayer near the physiological level of cholesterol comes from the susceptibility of membrane cholesterol to cholesterol oxidase (40–42) and the efficacy of cell fusion by viruses (43). There is also growing evidence that bilayer lipids associated with caveolae are in a special cholesterol-dependent state upon which the integrity of caveolar structure and many features of caveolar function, as well as their resistance to detergent extraction, critically depends (39, 44–46). Furthermore,

caveolae appear to be involved in cholesterol homeostasis (47–51). One could therefore imagine that these and/or other cholesterol-dependent bilayer domains are the sites from which signals of cholesterol abundance, in the form of cholesterol itself, are sent to the ER. Conversely, the feedback mechanism which tightly controls plasma membrane cholesterol could regulate the activity of caveolar constituents through cholesterol-dependent transitions in the bilayer (44). This hypothesis predicts that the activities of various informational proteins associated with cholesterol-dependent plasma membrane bilayer domains might be found to conform to the same J-curve demonstrated herein.

In conclusion, our data support three hypotheses regarding intracellular cholesterol homeostasis in human fibroblasts. The first is that cholesterol itself is the messenger that signals the needs of the plasma membrane to ER effectors. The second is that the acute and chronic adjustments made by fibroblasts to small changes in their cholesterol load are mediated by a thresholded dose–response relationship between the bulk and ER pools. The third is that this threshold may be set by a high-order bilayer transition which stimulates the transfer of plasma membrane cholesterol to the ER in proportion to the abundance of the cholesterol-dependent domains in the donor membranes.

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