The Influence of 25-Hydroxycholesterol on Phosphatidylinositol Content in Cultured Smooth Muscle Cells (43729)

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Abstract. **The effect of 25-hydroxycholesterol on phosphatidylinositol content was studied in subconfluent human arterial smooth muscle cells exposed to myo-[2-^{3}H]inositol for 90 min and then to 25-hydroxycholesterol at different concentrations or for different incubating periods. Ethanol at a concentration of 0.05% in culture medium was used as control. The results demonstrate that an increased incubation time or an increase in the concentration of 25-hydroxycholesterol produced an increase in phosphatidylinositol and its degraded products. The ratio of precursor to product at each stage of phosphatidylinositol turnover in 25-hydroxycholesterol-treated cells, however, was not different from that in control cells. The data indicate that the presence of 25-hydroxycholesterol in the media induced general activation of phosphatidylinositol synthesis and turnover.** [P.S.E.B.M. 1994, Vol 206]

Phosphatidylinositol, one of phospholipid constituents in plasma membranes, is intracellularly produced by direct reaction of cytidine diphosphate-diglyceride with inositol in the presence of cytidine diphosphate:diglyceride inositol phosphatidate transferase (1). Phosphatidylinositol is rapidly phosphorylated to form phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate by adding respectively one and two phosphates into the molecule at the 4 and 5 positions of inositol under the effect of phosphomonoesterase (1). Phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate can then be hydrolyzed in the presence of phospholipase C to form myo-inositol 1,4-bisphosphate and inositol 1,4,5-triphosphate which are effective regulators of cytosolic free Ca^{++} (2–4). Lysophosphatidylinositol, a metabolized product of phosphatidylinositol by phospholipase A_2, is a participant in the regulation of membrane fluidity (5). In studying phosphatidylinositol, Block *et al.* (6) found that low-density lipoprotein (LDL) stimulated the phosphatidylinositol cycle in smooth muscle cells of rats and concluded that the stimulatory effect came from general activation of phosphatidylinositol turnover. The effect of cholesterol oxidation products on phosphatidylinositol content and turnover has not been reported to date. 25-Hydroxycholesterol, an autoxidation product of cholesterol, may arise during the processing or cooking of foods containing cholesterol (7). We have found (8, 9) that 25-hydroxycholesterol enhanced Ca^{++} uptake and the cytosolic free Ca^{++} level in cultured cells. The altered membrane permeability may come from the insertion of 25-hydroxycholesterol into the membrane (10). Since 25-hydroxycholesterol has a strong binding affinity to oxysterol-binding protein (11) and the binding would affect the degradation of 3-hydroxy-3-methylglutaryl CoA reductase (12), we surmised that phosphatidylinositol content and turnover might also be affected by 25-hydroxycholesterol. We therefore investigated this proposed effect of 25-hydroxycholesterol in cultured human arterial smooth muscle cells.

Materials and Methods

25-Hydroxycholesterol obtained from Sigma Chemical Co. (St. Louis, MO) was dissolved in abso-

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lute ethanol at a concentration of 10 mg/ml, stored at −20°C under N_2 and diluted to experimental concentrations with Eagle's minimum essential medium (MEM, Sigma) supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, UT). The final concentration of ethanol in culture medium was 0.05%. Myo-[2-^{3}H]inositol (10–20 Ci/m mol, 370–740 GBq/m mol) was purchased from New England Nuclear Corp. (Boston, MA).

Cell Culture. Smooth muscle cells were obtained from arteries of human umbilical cords (13). The cells were cultured in 75-cm^2 tissue culture flasks (Corning Medical and Scientific Co., Park Ridge, IL) containing 8 ml of MEM supplemented with 20% FBS. The flasks were kept in a 5% CO_2 incubator at 37°C. The passage numbers of the cells used in this study ranged from 8 to 14.

Measurement of Content and Turnover of Phosphatidylinositol. Subconfluent cells in 75-cm^2 flask were exposed to 3 μCi of Myo-[2-^{3}H]inositol in 6 ml of MEM supplemented with 5% FBS (MEM-5%FBS) for 90 min. After the radioactivity-containing medium was aspirated, the cells were washed three times with MEM and then treated with 25-hydroxycholesterol (i) at a concentration of 5 μg/ml for 2, 4, 6, 24, and 48 hours and (ii) at concentrations of 0.1, 0.5, 1, and 5 μg/ml for 72 hours. MEM-5%FBS containing 0.05% ethanol was used as control. At the designated time, the cells were harvested and the total lipids were extracted by chloroform/methanol (2:1 v/v) (8). Free inositol, glycerophosphoinositol, myo-inositol 1-phosphate, myo-inositol 1,4-bisphosphate, and inositol 1,4,5-triphosphate in the water-soluble fraction were separated by anion-exchange chromatography and the concentrations were measured by radioactivity counting (14). At the same time, each eluting solution was counted as a blank. Inositol phospholipids, including phosphatidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate, and lysophosphatidylinositol, in the organic fraction were identified on TLC plates with the method of Billah and Lapetina (15). All spots and some silica gel on blank spaces were collected and counted. The results, in which nonspecific activity in the blank was already subtracted from sample radioactivity, were expressed in DPM/μg protein.

Statistical Analysis. All results are expressed as means ± SE. A multiple analysis of variance and the Scheffe test were used to determine the statistical significance.

Results

Table I shows that from 24 hr on the radioactivity in phosphatidylinositol was significantly higher in the cells treated with 25-hydroxycholesterol than that in control cells. When the cells were treated with 25-hydroxycholesterol at various levels, the increased radioactivity in phosphatidylinositol occurred along with enhanced concentrations of 25-hydroxycholesterol (Table II). These data indicate that the incubation of the cells with 25-hydroxycholesterol resulted in an increased phosphatidylinositol content. Moreover, a time-dependent decrease of phosphatidylinositol content in both 25-hydroxycholesterol-treated and control cells confirmed the degradation of phosphatidylinositol, as shown in Table I.

The increased phosphatidylinositol can come from a stimulated synthesis and/or an inhibited degradation. The degradation of phosphatidylinositol could proceed in four ways in which the degradation in the cells

Table I. The Time-Dependent Effect of 25-Hydroxycholesterol on Inositol Phospholipids in Cultured Human Arterial Smooth Muscle Cells from 2 to 48 hr

	hr				
	2	4	6	24	48
	DPM/μg protein				
Phosphatidylinositol					
Control (0.05% ethanol)	30 ± 4.0	30 ± 4.4	27 ± 5.7	16 ± 2.6	9.3 ± 1.3
25-Hydroxycholesterol	31 ± 4.4	32 ± 4.1	30 ± 4.2	21 ± 2.3[a]	16 ± 1.5[b]
Phosphatidylinositol 4-phosphate					
Control	0.31 ± 0.04	0.31 ± 0.05	0.31 ± 0.05	0.25 ± 0.02	0.21 ± 0.02
25-Hydroxycholesterol	0.27 ± 0.03	0.30 ± 0.03	0.29 ± 0.02	0.31 ± 0.03[a]	0.33 ± 0.03[b]
Phosphatidylinositol 4,5-bisphosphate					
Control	0.40 ± 0.05	0.40 ± 0.04	0.39 ± 0.05	0.36 ± 0.03	0.24 ± 0.02
25-Hydroxycholesterol	0.38 ± 0.06	0.40 ± 0.03	0.46 ± 0.05	0.39 ± 0.04	0.35 ± 0.02[b]
Lysophosphatidylinositol					
Control	1.1 ± 0.05	1.1 ± 0.05	1.1 ± 0.2	0.8 ± 0.03	0.5 ± 0.06
25-Hydroxycholesterol	1.3 ± 0.05	1.2 ± 0.03	1.0 ± 0.1	0.9 ± 0.03	0.7 ± 0.06[a]

Results are expressed as means ± SE of six experiments performed in duplicate. The superscripts represent statistical differences ([a]$P < 0.05$, [b]$P < 0.01$) compared with control group at the same incubating time. 25-Hydroxycholesterol was used at a level of 5 μg/ml medium. The range of DPM for this experiment was from 450 to 31310.

Table II. The Concentration-Dependent Effect of 25-Hydroxycholesterol on Inositol Phospholipids in Cultured Human Arterial Smooth Muscle Cells Treated with 25-Hydroxycholesterol for 72-hr

	μg 25-Hydroxycholesterol/ml medium				
	0	0.1	0.5	1.0	5.0
	DPM/μg protein				
Phosphatidylinositol	2.1 ± 0.1	2.4 ± 0.1	2.8 ± 0.1[b]	2.9 ± 0.1[b]	4.4 ± 0.2[b]
Phosphatidylinositol 4-phosphate	0.12 ± 0.01	0.16 ± 0.01	0.18 ± 0.02[a]	0.18 ± 0.03	0.21 ± 0.02[a]
Phosphatidylinositol 4,5-bisphosphate	0.13 ± 0.01	0.17 ± 0.01	0.19 ± 0.02[a]	0.19 ± 0.01[b]	0.22 ± 0.02[b]
Lysophosphatidylinositol	0.20 ± 0.01	0.25 ± 0.01	0.27 ± 0.02[b]	0.26 ± 0.01[b]	0.34 ± 0.03[b]

Results are means ± SE of six experiments performed in duplicate. The superscripts represent statistical differences ([a]$P < 0.05$, [b]$P < 0.01$) compared with the group without treatment with 25-hydroxycholesterol. The range of DPM for this experiment was from 360 to 3390.

treated with 25-hydroxycholesterol was more active than that in control cells from 24 hr of treatment on (Table I and III) 25-hydroxycholesterol: (i) stimulated phosphorylation of phosphatidylinositol to form phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate; (ii) enhanced hydrolysis of phosphatidylinositol and its phosphorylated products to transform myo-inositol 1-phosphate, myo-inositol 1,4-bisphosphate, and inositol 1,4,5-triphosphate; (iii) assisted the breakdown of phosphatidylinositol leading to the elevation of lysophosphatidylinositol and glycerophosphoinositol; and (iv) accentuated the degradation of phosphatidylinositol down to free inositol. These effects of 25-hydroxycholesterol on phosphatidylinositol turnover were enhanced with an increase in concentration of 25-hydroxycholesterol (Table II and IV). When the ratios of phosphatidylinositol 4-phosphate:phosphatidylinositol, myo-inositol 1-phosphate: phosphatidylinositol and lysophosphatidylinositol: phosphatidylinositol were calculated, no difference in any ratios was noted between 25-hydroxycholesterol-treated and control cells.

Discussion

Evidence for oxysterols' regulating effects on phospholipid concentration in cells has been found in previous studies (8, 16). 26-Hydroxycholesterol could stimulate an interconversion among phospholipids (16). The present study indicates that 25-hydroxycholesterol exerted an enhancing effect on the concentrations of both phosphatidylinositol and its degraded products. The ratios of precursor to product at the same stage of phosphatidylinositol turnover in the pair of 25-hydroxycholesterol-treated and control cells were not significantly different. This lack of significance infers that 25-hydroxycholesterol had no specific stimulating effect on phosphatidylinositol degradation. The increased phosphatidylinositol and the de-

Table III. The Time-Dependent Effect of 25-Hydroxycholesterol on Water-Soluble Inositol Content in Cultured Human Arterial Smooth Muscle Cells from 2 to 48 hr

	hr				
	2	4	6	24	48
	DPM/μg protein				
Inositol					
Control (0.05% ethanol)	5.4 ± 0.5	5.5 ± 0.5	5.7 ± 0.6	3.6 ± 0.3	2.8 ± 0.2
25-Hydroxycholesterol	5.8 ± 0.7	5.9 ± 0.6	5.1 ± 0.3	4.3 ± 0.5	3.5 ± 0.3
Glycerophosphoinositol					
Control	2.7 ± 0.4	2.8 ± 0.3	2.9 ± 0.4	2.5 ± 0.3	1.9 ± 0.2
25-Hydroxycholesterol	2.8 ± 0.3	3.2 ± 0.4	3.1 ± 0.2	3.1 ± 0.5[a]	2.8 ± 0.3[a]
Myo-inositol 1-phosphate					
Control	2.8 ± 0.4	2.6 ± 0.4	2.8 ± 0.5	2.8 ± 0.3	2.0 ± 0.2
25-Hydroxycholesterol	2.9 ± 0.4	2.9 ± 0.3	3.2 ± 0.2	3.1 ± 0.5	2.8 ± 0.3[a]
Myo-inositol 1,4-bisphosphate					
Control	2.7 ± 0.2	2.6 ± 0.3	2.7 ± 0.2	2.5 ± 0.3	2.0 ± 0.2
25-Hydroxycholesterol	2.8 ± 0.4	2.7 ± 0.3	2.9 ± 0.3	3.1 ± 0.3[a]	2.9 ± 0.2[a]
Inositol 1,4,5-triphosphate					
Control	2.7 ± 0.2	2.4 ± 0.3	2.6 ± 0.2	2.6 ± 0.2	2.0 ± 0.2
25-Hydroxycholesterol	2.4 ± 0.2	2.6 ± 0.4	3.1 ± 0.3	3.0 ± 0.3[a]	2.9 ± 0.3[a]

Results are expressed as means ± SE of six experiments performed in duplicate. The superscripts represent statistical differences ([a]$P < 0.05$, [b]$P < 0.01$) compared with control group at the same incubating time. 25-Hydroxycholesterol was used at a level of 5 μg/ml medium. The range of DPM for this experiment was from 670 to 1460.

Table IV. The Concentration-Dependent Effect of 25-Hydroxycholesterol on Water-Soluble Inositol Content in Cultured Human Arterial Smooth Muscle Cells Treated with 25-Hydroxycholesterol for 72 hr

	μg 25-Hydroxycholesterol/ml medium				
	0	0.1	0.5	1.0	5.0
	DPM/μg protein				
Inositol	1.6 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	2.6 ± 0.1[b]
Glycerophosphoinositol	1.4 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	2.2 ± 0.1[b]
Myo-inositol 1-phosphate	1.3 ± 0.1	1.5 ± 0.1[a]	1.5 ± 0.1[a]	1.6 ± 0.1[b]	2.1 ± 0.1[b]
Myo-inositol 1,4-bisphosphate	1.3 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.1[a]	2.0 ± 0.1[a]
Inositol 1,4,5-triphosphate	1.3 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	2.0 ± 0.1[b]

Results are means ± SE of six experiments performed in duplicate. The superscripts represent statistical differences ([a]$P < 0.05$, [b]$P < 0.01$) compared with the group without treatment with 25-hydroxycholesterol. The range of DPM for this experiment was from 430 to 970.

graded products, therefore, might result from the fact that 25-hydroxycholesterol generally activates cellular phosphatidylinositol synthesis and turnover, though the mechanism of that activation needs to be further investigated. Evidence supporting our results can be found in two previous studies (6, 17): (i) LDL stimulated the phosphatidylinositol cycle in several cell lines, including human platelet, human lymphocyte, human fetal lung fibroblast, and rat smooth muscle cell from aorta (6); and (ii) certain oxysterols, including 25-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, cholesterol α- and β-epoxides were contained in LDL (17). Given these previous findings and our present data, we cannot exclude the possibility of an effect of oxysterols in LDL on phosphatidylinositol cycle.

There are at least two possible effects of 25-hydroxycholesterol responsible for the increased synthesis and turnover of phosphatidylinositol. First, 25-hydroxycholesterol was shown to be able to insert itself into erythrocyte membranes and the insertion could induce a disturbance of plasma membrane (18). The incorporation of 0.5 mole% 25-hydroxycholesterol into liposomes substantially increased the permeability of liposomes to Ca^{++} (19), whereas the presence of cholesterol at 10 mole% did not show any effect on permeability (19). This implies that only a small amount of oxidized sterol is needed to perturb membranes. Second, a decrease in the cholesterol: phospholipid ratio by 25-hydroxycholesterol, via an inhibition of cholesterol synthesis (12), could also disturb the plasma membrane. The disturbed plasma membrane could then affect the characteristics of proteins, including enzymes and receptors (20).

25-Hydroxycholesterol has been exhibited to stimulate Ca^{++} influx into cells (21, 22). In the present study, we found that 25-hydroxycholesterol accelerated the degradation of phosphatidylinositol. The degraded products might play a role when 25-hydroxycholesterol stimulated Ca^{++} influx. Lysophosphatidylinositol, which has been reported to alter membrane fluidity (5), had a higher level in the cells treated with 25-hydroxycholesterol than that in control cells in this study. Since Ca^{++} influx can be affected by membrane structure and permeability (5, 22), the stimulating effect of 25-hydroxycholesterol on Ca^{++} uptake could be, at least in part, related to this higher level of lysophosphatidylinositol. Though the metabolic products of phosphatidylinositol are minor constituents of cellular lipid (23), they are crucial to the alteration of cytosolic free Ca^{++} concentration (2–4). For instance, inositol 1,4,5-triphosphate enhanced cytosolic free Ca^{++} level through both a stimulation of Ca^{++} influx and a faster mobilization of Ca^{++} from intracellular stores. The mobilization was obviously energized by an activation of phosphatidylinositol metabolism in smooth muscle cells cultured in Ca^{++}-free medium (24). Moreover, since phosphatidylinositol and its two phosphorylated products are potent activators of ATPase (3), whereas inositol 1,4,5-triphosphate is an inhibitor (4), the ATP pump could be regulated by activating/deactivating dynamics between the stimulator and the inhibitor. Therefore, we suggest that the increasing effect of 25-hydroxycholesterol on cytosolic free Ca^{++} should involve more than one factor.

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1. Holub BJ. The cellular forms and functions of the inositol phospholipids and their metabolic derivatives. Nutr Rev **45**:65–71, 1987.
2. Irvine RF. Inositol phosphates and Ca^{++} entry: Toward a proliferation or a simplification? FASEB J **6**:3085–3091, 1992.
3. Carafoli E, Zurini M. The Ca^{++}-pumping ATPase of plasma membranes purification, reconstitution and properties. Biochim Biophys Acta **683**:279–301, 1982.
4. Choquette D, Hakin G, Filoteo AG, Plishker GA, Bostwick GR, Penniston JT. Regulation of plasma membrane Ca^{2+} ATPase by lipids of the phosphatidylinositol cycle. Biochem Biophys Res Commun **125**:908–915, 1984.
5. Cooper RA. Influence of increased membrane cholesterol on

membrane fluidity and cell function in human red blood cells. J Supramol Structure **8**:413–430, 1978.
6. Block LH, Knorr M, Vogt E, Locher R, Vetter W, Groscurth P, Qiao B, Pometta D, James R, Regenass M, Pletscher A. Low density lipoprotein causes general cellular activation with increased phosphatidylinositol turnover and lipoprotein catabolism. Proc Natl Acad Sci USA **85**:885–889, 1988.
7. Kumar N, Singhal OP. Cholesterol oxides and atherosclerosis: A review. J Sci Food Agric **55**:497–510, 1991.
8. Zhou Q, Jimi S, Smith TL, Kummerow FA. The effect of 25-hydroxycholesterol on accumulation of intracellular calcium. Cell Calcium **12**:467–476, 1991.
9. Kawamura K, Kummerow FA. Effect of 25-hydroxycholesterol on cytotoxicity and prostacyclin production in cultured human umbilical arterial endothelial cells. Eicosanoids **5**:29–34, 1992.
10. Smith LL, Johnson BH. Biological activities of oxysterols. Free Rad Biol Med **7**:285–332, 1989.
11. Taylor FR, Kandutsch AA. Oxysterol binding protein. Chem Phys Lipids **38**:187–194, 1985.
12. Taylor FR. Correlation among oxysterol potencies in the regulation of the degradation of 3-hydroxy-3-methylglutaryl CoA reductase, the repression of 3-hydroxy-3-methylglutaryl CoA synthase and affinities for the oxysterol receptor. Biochem Biophys Res Commun **186**:182–189, 1992.
13. Zhou Q, Smith TL, Kummerow FA. Cytotoxicity of oxysterols on cultured smooth muscle cells from human umbilical arteries. Proc Soc Exp Biol Med **202**:75–80, 1993.
14. Berridge MJ, Dawson RMC, Downes CP, Heslop JP, Irvine RF. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem J **212**:473–482, 1983.
15. Billah MM, Lapetina EG. Rapid decrease of phosphatidylinositol 4,5-bisphosphate in thrombin-stimulated platelets. J Biol Chem **257**:12705–12708, 1982.
16. Kou I-L, Pikul J, Kummerow FA. Influence of 26-hydroxycholesterol on the composition and function of gel-filtered platelets. J Am Coll Nutr **10**:114–122, 1991.
17. Addis PB, Emanuel HA, Bergmann SD, Zavoral JH. Capillary GC quantification of cholesterol oxidation products in plasma lipoproteins of fasted humans. Free Radical Biol Med **7**:179–183, 1989.
18. Szostek R, Kucuk O, Lis LJ, Tracy D, Mata R, Dey T. Kauffman JW, Yachnin S, Westerman MP. Effect of inserted oxysterols on phospholipid packing in normal and sickle red blood cell membranes. Biochem Biophys Res Commun **180**:730–734, 1991.
19. Holmes RP, Yoss NL. 25-Hydroxycholesterols increase the permeability of liposomes to Ca^{++} and other cations. Biochim Biophys Acta **770**:15–21, 1984.
20. Holmes R, Kummerow FA. Membrane perturbations in atherosclerosis. In: Aloia RC, Boggs JM, Eds. Membrane Fluidity in Biology. Boca Raton: Academic Press, pp281–305, 1985.
21. Boissonneault GA, Heiniger H-J. 25-Hydroxycholesterol-induced elevation in $^{45}Ca^{++}$ uptake: Correlation with depressed DNA synthesis. J Cell Physiol **120**:151–156, 1984.
22. Boissonneault GA, Heiniger H-J. 25-Hydroxycholesterol-induced elevations in $^{45}Ca^{++}$ uptake: Permeability changes in P815 cells. J Cell Physiol **125**:471–475, 1985.
23. Michell RH. Inositol phospholipids and cell surface receptor function. Biochim Biophys Acta **415**:81–147, 1975.
24. Berridge MJ. Inositol triphosphate and diacylglycerol: Two interacting second messengers. Annu Rev Biochem **56**:159–193, 1987.