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Lovastatin Increases Surface Low Density Lipoprotein Receptor Expression by Retarding the Receptor Internalization Rate in Proliferating Lymphocytes

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We examined the effects of Lovastatin on LDL receptor (LDL-R) expression and rate of internalization in interleukin-2 (IL-2) expanded phytohemagglutinin-stimulated lymphocytes. Lovastatin increased the surface LDL-R expression, but not DiI-LDL uptake, by up to 30% regardless of whether cell proliferation was affected. It caused a dose-dependent reduction in the LDL-R internalization rate as determined with monensin. Lovastatin had no effect on IL-2 receptor internalization. Inhibition of DNA synthesis by hydroxyurea or protein tyrosine kinase activity by genistein failed to affect the LDL-R internalization rate. Co-incubation of cells with Lovastatin and mevalonate or LDL completely restored the rate of LDL-R internalization. We conclude that Lovastatin increases the apparent surface LDL-R expression by retarding the rate of LDL-R internalization. The effect is mediated through the mevalonate pathway but not the anti-mitogenic property of Lovastatin.

Lovastatin is a potent competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase which catalyses the rate-limiting conversion of HMGCoA to mevalonate in the cholesterol synthetic pathway (1,2). In human and animal studies, Lovastatin treatment is associated with 30-50% reduction in serum total and low density lipoprotein (LDL) cholesterol levels (3-5). This cholesterol lowering effect of Lovastatin is attributed to increases in VLDL catabolism (6), decreases in LDL production (7) as well as increases in specific receptor-mediated uptake in the liver (8-10). In peripheral tissue cells such as circulating mononuclear cells, Lovastatin increases both the LDL receptor (LDL-R) mRNA level and activity (11-13).

Lovastatin, by virtue of its inhibitory effects on the mevalonate pathway, has also been shown to inhibit growth factor-/mitogen-induced DNA synthesis and cell proliferation (14-16), arresting cells in G1 (17,18) phase, and to suppress tumour growth in animals (19,20). Since LDL-R gene transcription and activity are increased in proliferating cells (21,22), it is anticipated that Lovastatin will attenuate the LDL-R expression by inhibiting cell proliferation. However, we previously reported that Lovastatin increased the surface expression of LDL-R in proliferating lymphocytes (23). In the current study, we examined the effects of Lovastatin on the LDL-R expression in interleukin-2 (IL-2) expanded phytohemagglutinin-stimulated lymphocytes, and report here that Lovastatin increases the surface LDL-R expression by decreasing the rate of internalization of the receptor.

MATERIALS AND METHODS

Mononuclear cell isolation and culture. Mononuclear cells were isolated over Ficoll-paque (Pharmacia) using a modified procedure of Boyum (23,24). The cells were then cultured at a concentration of 1×10^6 cells/mL ($0.4\times10^6/cm^2$) for 68 hours at 37°C in 5% CO_2 and RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1% phytohemagglutinin (PHA-M, Sigma) and 10% human lipoprotein deficient serum (LPDS) prepared from pooled human plasma as described (25). For maximum expression of LDL-R, PHA-stimulated lymphocytes were washed and resuspended at a density of 0.2×10^6/mL in RPMI supplemented with 10% LPDS and 50 units/mL of IL-2 (Cetus Corporation, USA). In some experiments, the indicated amount of Lovastatin (Merck Frosst Canada Inc.), hydroxyurea, mevalonate or genistein (Sigma, USA) was added. The cells were then incubated in 5% CO_2 at 37°C for another 19 hours. For measurement of receptor internalization rate, the change in surface LDL-R and/or IL2-R expression were monitored in the presence and absence of monensin (25 μM or otherwise stated) which prevents the internalized receptors from returning to the surface (26,27) for 25 minutes (or otherwise indicated). An equivalent amount of methanol (not exceeding 0.25% v/v) was added to cultures that did not receive monensin. The receptor internalization rate is reflected by the percentage of change in surface expression in the presence to that in the absence of monensin over the specified period.

Dual color flow cytometry for surface expression of LDL-R and IL2-R. Surface expression of LDL-R and IL2-R were determined by indirect dual color flow cytometry as described (23), using a specific mono-

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clonal antibody to LDL-R (Amersham), a phycoerythrin conjugated goat anti-mouse antibody (Jackson ImmunoResearch Inc., USA) and a fluorescein labelled IL2-R antibody (Becton-Dickinson, USA). Ten thousand cells from each sample were analyzed on an FACScan flow cytometer (Becton-Dickinson, Mountainview, California, USA), and the fluorescence readings were analyzed using the LYSYS II program (Becton-Dickinson) to give a mean fluorescence intensity (per cell) in arbitrary units. Background fluorescence due to cell autofluorescence and nonspecific binding of isotype-matched control antibodies was subtracted to give a net mean fluorescence (MF) which reflects the quantity of the respective receptors present on the surface.

Flow cytometric analysis of DiI-LDL uptake. Human LDL ($1.019 < \text{density} < 1.063$) was isolated from pooled plasma by density gradient ultracentrifugation (28) and was labelled with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes, Inc., USA) as described (29). The specific DiI-LDL uptake at 37°C provides a measure of the LDL-R activity (30,31) and was determined as described (23,29,32). Cell-associated fluorescence due to DiI (FL2) was analyzed on a FACScan flow cytometer as described in the previous paragraph. Nonspecific uptake of DiI-LDL was assessed in the presence of 20-fold excess of LDL and specific uptake was calculated as the difference between total and nonspecific uptake.

Cell cycle analyses by propidium iodide. Harvested cells were washed once with PBS, fixed in 67% ethanol in PBS (2:1 v/v) and stored at 4°C until analysis. On the day of analysis, $1\text{-}1.5\times10^6$ ethanol-fixed cells in a 12×75 mm round bottom tube were washed once with PBS and treated with RNAse to remove any double stranded RNAs before final suspension in 50 μg/mL of Propidium Iodide (Sigma, USA) in PBS, pH 7.4. Cellular fluorescence (FL2) from 15,000 cells was routinely collected in a flow cytometer. The distribution of DNA was analyzed by the CELLFit program (Becton Dickinson) to calculate, based on their respective DNA content, the percentage of cells in G_0G_1, S and G_2M phases. Stimulation index (SI), calculated as $(S + G_2M)/G_0G_1$, was taken as a measure of the degree of cell activation and proliferation (33,34).

Statistics. Results are expressed as mean±SE. Sample means are compared using the Student's t test and are considered significant if $p<0.05$.

RESULTS

Effects of Lovastatin on the surface expression of LDL-R and DiI-LDL uptake. As shown in Figure 1, addition of as little as 0.1 μmol/L of Lovastatin caused a significant 31.0±4.2% (mean±SE) increase in surface LDL-R expression (paired t test, $p<0.01$). This level of LDL-R expression remained similar as the concentration of Lovastatin increased to 2.5 μM. Further increase of Lovastatin concentration to 5 μM resulted in a small reduction in the surface LDL-R expression which was still 16.6±1.8% higher than controls that were not treated with Lovastatin. The DiI-LDL uptake, however, was not increased by Lovastatin. The DiI-LDL uptake decreased steadily as the concentration of Lovastatin increased from 0.1 to 2.5 μM. At 5 μM, Lovastatin caused a significant 13.8±1.2% reduction in DiI-LDL uptake when compared to cultures not treated with Lovastatin (paired t test, $p<0.01$).

The effects of Lovastatin on cell proliferation as reflected by the IL2-R expression and cell cycle phase distribution are shown in Table 1. Lovastatin at 1 μM did not significantly affect cell proliferation as indicated by the proportion of cells in dividing phases (S and G_2M) and the surface IL2-R expression (paired t test, $p>0.05$). At 5 μM, Lovastatin significantly decreased the IL2-R expression by 19.9% and increased the proportion of cells in G0G1 from 47.0% to 52.4% (paired t test, $p<0.05$).

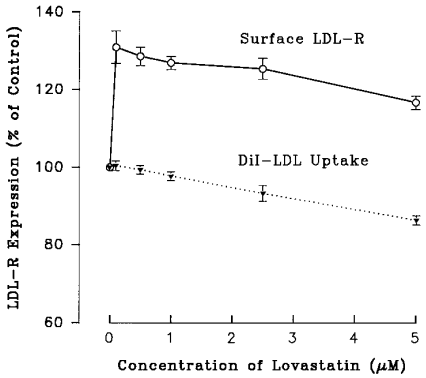

FIG. 1. Dose-Effects of Lovastatin on surface expression of LDL-R and DiI-LDL Uptake. Surface Low Density Lipoprotein Receptor (LDL-R) expression and DiI-LDL uptake were measured as described in Methods in IL-2 expanded PHA-stimulated lymphocytes supplemented with 0-5 μM Lovastatin for 19 hours. Values are expressed as percentages of that obtained from cultures without Lovastatin (control). Error bars signify ± SE of duplicate measurements from two separate experiments.

Effects of monensin on the cell surface expression of LDL-R. To examine the LDL-R internalization rate, the change in surface LDL-R expression in the presence of monensin which blocks the return of receptors to the surface was determined. As shown in Figure 2A, addition of monensin to IL-2 expanded PHA-stimulated lymphocytes for 25 minutes caused a dose-dependent reduction of the surface LDL-R expression, reaching a maximum reduction of 64% at about 10 μM. Increasing the concentration of monensin to 30 μM did not decrease the LDL-R expression further. The time course of the LDL-R reduction in the presence of 25 μM monensin is presented in Figure 2B. The surface LDL-R expression decreased by 55% in 10 minutes, and up to 65% in 30 minutes. Twenty five μmol/L of monensin for 25 minutes was chosen for all subsequent experiments.

Effect of Lovastatin on LDL-R internalization rate. Figure 3 shows the effect of Lovastatin on the LDL-R internalization rate measured as the percentage decrease in surface LDL-R expression in the presence of 25 μM of monensin over 25 minutes. In cultures that have not been treated with Lovastatin, a reduction in surface LDL-R expression of 56.3±1.3% (mean±SE) in 25 minutes was observed. When the cells were preincubated with increasing concentrations of Lovastatin for 19 hours, a dose-dependent attenuation of the LDL-R internalization rate was observed; the LDL-R internal-

TABLE 1

Effects of Various Treatments on Surface LDL-R Expression and Cell Proliferation

Treatment	LDL-R (MF)	IL2-R (MF)	Cell cycle phases			SI
			G_0G_1	S	G_2M	
Nil	479.5 ± 10.7	565.8 ± 14.5	47.0 ± 0.8	42.5 ± 1.3	10.4 ± 1.1	1.13 ± 0.04
+ 1 μM Lovastatin	621.1 ± 15.1*	544.9 ± 6.8	47.2 ± 1.0	41.5 ± 1.1	11.3 ± 0.8	1.12 ± 0.05
+ 5 μM Lovastatin	560.7 ± 12.8*	453.1 ± 8.6*	52.4 ± 1.2*	35.2 ± 1.2*	12.4 ± 0.6	0.91 ± 0.04*
+ 0.2 mM Hydroxyurea	281.9 ± 8.1*	526.1 ± 14.6	46.3 ± 0.7	46.9 ± 1.5	6.8 ± 0.9	1.16 ± 0.03
+ 1.0 mM Hydroxyurea	216.6 ± 8.7*	424.6 ± 6.8*	93.6 ± 1.4*	4.5 ± 0.2*	1.9 ± 0.2*	0.07 ± 0.01*
+ 15 μg/mL Genistein	267.2 ± 6.8*	541.5 ± 11.0	48.0 ± 0.8	41.2 ± 0.8	10.9 ± 0.9	1.08 ± 0.04

Note. LDL-R: LDL receptor; IL2-R: Interleukin-2 receptor; MF: Mean Fluorescence; SI: Stimulation Index; "*": Statistically significant (unpaired t test, $p<0.01$) when compared to control that received no treatment. Values represent mean ± SE from 3-8 determinations.

ization rate decreased significantly from 56.3±1.3% in the absence of Lovastatin to 11.5±2.0% at 5 μM Lovastatin (paired t test, $p<0.001$). The mean fluorescence of surface IL2-R, which was measured simultaneously as the LDL-R, increased 8-16% over the 25-minute monensin treatment. The increase was accompanied by a 10-15% increase in cell size as reflected by the forward scatter (FSC) (results not shown). The increase in surface IL2-R expression, however, did not change significantly over the range of Lovastatin concentrations tested.

Effects of hydroxyurea and genistein. To examine the possible mechanisms involved in the effects of Lovastatin on LDL-R internalization, lymphocyte cultures were treated with hydroxyurea, a DNA synthesis inhibitor, and genistein, a protein tyrosine kinase inhibitor as described in Methods. Figure 4 shows that addition of up to 1.0 mM hydroxyurea did not significantly affect the LDL-R internalization rate (unpaired t test, $p>0.05$). A slight accumulation of IL2-R on the cell surface, which did not change over the concentrations of hydroxyurea, was also observed. Despite the lack of effect on LDL-R internalization, hydroxyurea caused a significant 38.8% reduction in the surface expression of LDL-R at a concentration (0.2 mM) that did not affect cell cycle distribution significantly (see Table 1). Increasing the hydroyxurea concentration to 1 mM caused a further 14.2% reduction in the surface expression of LDL-R and arrested almost every cell (94%) in G0G1 phase, reducing the SI from 1.11 in the absence of hydroxyurea to 0.07. The IL2-R expression was also significantly reduced by 25% (unpaired t test, $p<0.01$).

Similarly, addition of 15 μg/mL genistein to the cell cultures for 19 hours did not affect the LDL-R internal-

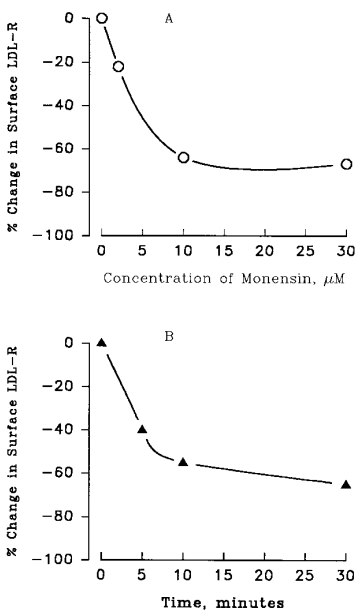

FIG. 2. Reduction of Surface LDL-R in the presence of Monensin. (A) Reduction of surface LDL-R over 0-30 μM monensin in 25 minutes. Results are expressed as a percentage of the control culture (without monensin) which was taken as 100%. (B) Percent change in surface LDL-R expression in the presence of 25 μM monensin over time. Results represent the mean of duplicate measurements.

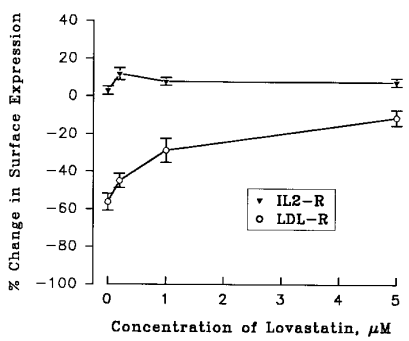

FIG. 3. Effect of Lovastatin on the LDL-R Internalization Rate. IL-2 expanded PHA-stimulated lymphocytes were incubated with 0-5 μM of Lovastatin for 19 hours. Monensin (25 μM) was added during the last 25 minutes of culture. Control cultures received the same amount of methanol (solvent for monensin). Values for IL2-R (▼) and LDL-R (○) are expressed as a percentage change of controls. Each value represents the mean±SE of triplicate determinations from two separate experiments.

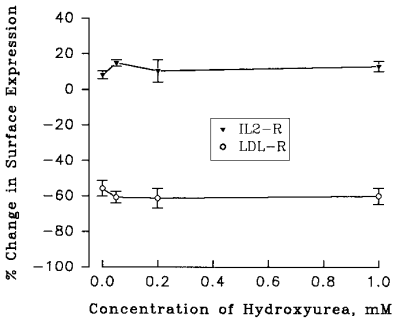

FIG. 4. Effects of Hydroxyurea. IL-2 expanded PHA-stimulated lymphocytes were incubated with 0-1 mM of hydroxyurea for 19 hours. Twenty five minutes prior to dual color detection of IL2-R and LDL-R, 25 μM monensin was added. Control cultures received the same amount of methanol (solvent for monensin). Values for IL2-R (▼) and LDL-R (○) are expressed as a percentage change of that obtained with controls. Each value represents the mean±SE of triplicate determinations from two separate experiments.

ization rate significantly, 60.8±1.9% versus 56.3±1.3% (unpaired t test, $p>0.05$). On the other hand, the surface LDL-R expression was reduced significantly by 42% (unpaired t test, $p<0.001$) (see Table 1). The IL2-R expression and cell cycle distribution were not affected significantly.

Restoration of LDL-R internalization rate by mevalonate and LDL. Figure 5 shows the effects of mevalonate and LDL on the restoration of LDL-R internalization caused by Lovastatin. Preincubation of the cultures with 10 mM mevalonate concurrently with 1 μM Lovastatin completely abrogated the effect of Lovastatin, increasing the LDL-R internalization rate from 28.8±1.9% (Lovastatin alone) to more than 56.4±3.6% (Lovastatin plus mevalonate). Alternatively, incubation of 20 μg/mL LDL 30 minutes before the addition of monensin in cells treated with 1 μM of Lovastatin also allowed complete restoration of the LDL-R internalization rate i.e. increase from 28.8±1.9 to 61.0±1.7%.

DISCUSSION

Low density lipoprotein receptor (LDL-R) is a surface glycoprotein that is responsible for the binding and uptake of LDL. It is upregulated by sterol deprivation as well as cell activation and proliferation (21,22,35). Lovastatin, an HMG-CoA reductase inhibitor that increases LDL-R mRNA and activity by attenuating endogenous cholesterol synthesis, is also an inhibitor for cell proliferation (17-19). In proliferating cells, much of the LDL-R upregulating effect of Lovastatin is expected to be attenuated due to its inhibition of cell proliferation. However, we previously observed that Lovastatin increased the surface LDL-R expression in proliferating lymphocytes (23). Since there is no significant storage or reservoir for the receptor within the cytoplasm (36), an increase in surface expression of the receptor implies either an actual increase in receptor number or a preferential distribution of the receptor on cell surface. In the present study, we showed that the Lovastatin-induced increase in surface LDL-R expression was caused by a reduction in the receptor internalization rate, an effect of Lovastatin that, to our knowledge, has not been described previously.

In IL-2 expanded PHA-stimulated lymphocytes, more than 50% of the cell population were in dividing phases (S and G2M) (see Table 1), and the surface LDL-R expression was some 10-fold higher than freshly isolated lymphocytes (results not shown). Treating the cells with Lovastatin up to 5 μM caused significant increases in surface LDL-R expression regardless of whether cell proliferation was attenuated. The increase was significantly lower at high Lovastatin concentration where cell proliferation as reflected by IL2-R expression and SI was significantly impeded. The increase in surface LDL-R expression, however, was not paralleled by similar increase in DiI-LDL uptake, indicating a lack of actual increase in LDL-R activity (Figure 1). At high Lovastatin concentration (e.g. 5 μM), there was even a small but significant drop in DiI-LDL uptake. These results indicate that attenuating cell proliferation reduces LDL-R activity. At low concentrations of Lovastatin (1 μM or below) where cell proliferation was not significantly affected, no change in LDL-R activity (DiI-LDL uptake) was observed. Since Lovastatin stimulates the upregulation of LDL-R mRNA and activity in quiescent cells (11,12), whether this lack of stimulation of LDL-R activity by Lovastatin represents a reduction in the sensitivity of the proliferating cells towards the stimulating effect of Lovastatin is not clear.

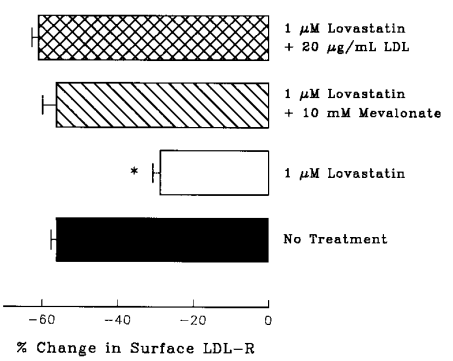

FIG. 5. Restoration of LDL-R Internalization Rate by Mevalonate and LDL. The percentage change in surface LDL-R in the presence of 25 μM monensin for 25 minutes was determined as described in Methods. Cultures received (1) no treatment, (2) 1 μM Lovastatin, (3) 1 μM Lovastatin + 10 mM mevalonate and (4) 1 μM Lovastatin + 20 μg/mL LDL added 30 minutes before the addition of monensin. Error bars signify SE from triplicate measurements from 2 separate experiments. "*" indicates significant difference from each of the three data groups (paired t test, $p<0.01$).

As the lack of increase in DiI-LDL uptake argues against any actual increase in LDL-R activity (or number) under the influence of Lovastatin, the observed increase in surface expression is likely to be due to preferential distribution on cell surface. Using a monovalent carboxylic ionophore, monensin, to block the internalized as well as newly synthesized LDL-R from migrating to the plasma membrane from the Golgi (37,38), we measured the rate of LDL-R internalization by monitoring the changes in surface LDL-R expression over 25 minutes. In the presence of monensin, about 56% of LDL-R entered the cell. This rate of internalization is quite comparable to that reported in fibroblast (27). When Lovastatin was added, a dose-dependent decrease in the LDL-R internalization rate was observed; at 0.2 μM Lovastatin, 45% of LDL-R disappeared from the cell surface within 25 minutes while at 5 μM, only 11.5% of LDL-R entered the cell (Figure 3).

Since Lovastatin has been shown to inhibit DNA synthesis (15-18) and to reduce the level of protein tyrosine phosphorylation in the β subunit of the insulin receptor (39), we examined whether its effect on LDL-R internalization could be mediated by inhibition of DNA synthesis or protein tyrosine kinase activity. Incubation with different concentrations of hydroxyurea, an inhibitor of DNA synthesis (40), caused a dose-dependent reduction in the surface LDL-R expression (see Table 1) but did not affect its internalization rate (Figure 4) regardless of whether IL2-R expression or SI was affected. Similarly, incubation of 15 μg/mL genistein, a protein tyrosine kinase inhibitor (41), decreased the surface LDL-R expression but did not change the LDL-R internalization rate, nor the IL2-R expression or SI significantly. Apparently, the attenuation of LDL-R internalization by Lovastatin does not appear to be mediated through inhibition of DNA synthesis or tyrosine kinase activity.

We next examined if the effect of Lovastatin could be reversed by releasing the blockade of the mevalonate pathway by supplementing the cultures with mevalonate or LDL. Addition of 10 mM of mevalonate to Lovastatin-treated cultures completely restored the LDL-R internalization rate from 28.8% to 56.4% (see Figure 5). Furthermore, preincubation of LDL for just 30 minutes prior to internalization assay also abrogated the Lovastatin effect on LDL-R internalization. These results suggest a role of cholesterol or its metabolites in the action of Lovastatin in reducing the LDL-R internalization rate. In addition, Lovastatin slows down the spontaneous or constitutive internalization of unoccupied LDL-R but not ligand-bound receptors. Since about 70% of the LDL-R are normally associated with clathrin-coated pits (42) (a prerequisite for LDL-R to be internalized) which make up only 2% of the cell surface, any localization of the receptors outside the coated pits may lead to a reduction in receptor internalization. In cells from FH patients with an internalization defect, LDL-R has been reported to be scattered on the cell surface rather than clustered in coated pits (43). Receptors such as the insulin receptor and the EGF receptor that do not recycle spontaneously in the absence of bound ligand are normally located outside the clathrin-coated pits (44,45). Lovastatin may disrupt the normal transfer of LDL-R onto clathrin coated pits and cause them to reside outside these pits, thereby reducing their ability to enter the cell spontaneously. This theory of LDL-R misplacement may also explain why an apparent increase in surface LDL-R was observed despite no significant changes occurred in DiI-LDL uptake when the cells were treated with Lovastatin. If Lovastatin affects other parts of the internalization pathway such as the assembly of cytosolic clathrin or the recruitment of the adaptor proteins to plasma membrane, the uptake of DiI-LDL will also be affected, a phenomenon that is not observed in this study.

Receptor mediated endocytosis is a very important biological phenomenon. It not only represents a common pathway for macromolecules such as LDL, transferrin, transcobalamin II, yolk proteins, EGF, insulin, chorionic gonadotropin, nerve growth factor, asialoglycoprotein, α-2-macroglobulin, certain viruses and toxins (46) to enter a mammalian cell but also determines what metabolic consequences these molecules may bring. It will be interesting to know if the Lovastatin also affects any of these receptors. In many receptors, especially those that do not recycle, an increase in residence time on the cell surface will mean a longer life span to carry out its biological function. Understanding the mechanisms by which Lovastatin exerts its effects on LDL-R may lead to a better understanding of how the entry of many macromolecules and therefore their biological functions can be regulated.

In summary, we have presented evidence that Lovastatin increases the apparent surface LDL-R expression by retarding the LDL-R internalization in proliferating lymphocytes. The effect appears to be mediated through the mevalonate pathway but not the anti-mitogenic properties of Lovastatin.

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