

Mitogenic stimulation alters the regulation of LDL receptor gene expression in human lymphocytes

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Abstract To address the possibility that influences other than ambient cholesterol concentrations regulate low density lipoprotein (LDL) receptor expression, the effect of mitogenic activation on the levels of LDL receptor mRNA in human lymphocytes was examined. Mitogenic stimulation of freshly isolated human peripheral blood mononuclear cells (PBMC) cultured in medium containing saturating concentrations of LDL resulted in cell cycle entry as evidenced by increased levels of mRNA for the interleukin-2 receptor, and also increased LDL receptor mRNA levels by 9-fold. Whereas LDL receptor gene expression was also induced when resting control PBMC were incubated in lipoprotein-deficient medium, mitogenic activation of PBMC cultured in the absence of LDL stimulated a further 3-fold increase in LDL receptor mRNA levels. The increase in LDL receptor mRNA levels in mitogen-stimulated PBMC was dependent on continued protein synthesis, was not the result of mRNA stabilization, and therefore most likely reflected enhanced gene transcription. It was unlikely that the increase in LDL receptor mRNA levels observed in mitogen-stimulated cells related merely to sterol deprivation since suppression of endogenous cholesterol synthesis with lovastatin increased LDL receptor mRNA only modestly. Moreover, mitogen-stimulated PBMC continued to synthesize cholesteryl esters, a storage form of cholesterol, confirming that they were not functionally deprived of sterols. Although mitogenic stimulation increased LDL receptor mRNA levels in PBMC, regulation by exogenous LDL was observed. Thus, LDL down-regulated LDL receptor gene expression in both control and mitogen-stimulated PBMC. Down-regulation was less effective in the latter; however, LDL down-regulated endogenous sterol synthesis to an equivalent extent in both control and mitogen-stimulated PBMC. By contrast, the oxygenated sterol, 25-hydroxycholesterol, and mevalonate, the precursor of endogenously synthesized sterols, down-regulated LDL receptor mRNA levels comparably in mitogen-stimulated and control PBMC. These data indicate that mitogenic stimulation provides an additional stimulus for LDL receptor gene expression over and above that of ambient sterols and, therefore, suggest that signals transduced during cellular activation play a role in regulation of LDL receptor mRNA levels. —Cuthbert, J. A., and P. E. Lipsky. Mitogenic stimulation alters the regulation of LDL receptor gene expression in human lymphocytes. *J. Lipid Res.* 1990. 31: 2067-2078.

Supplementary key words LDL receptor • mRNA • peripheral blood mononuclear cells

When peripheral blood lymphocytes are stimulated with the mitogenic lectin phytohemagglutinin, the responding

T lymphocytes enter the cell cycle, divide, and then continue through multiple rounds of cell division. During each round of the cell cycle, new membrane is synthesized from cholesterol and phospholipid. The cholesterol may be obtained by endogenous synthesis from acetate or by uptake of exogenous cholesterol in lipoproteins via specific receptors for low density lipoprotein (LDL) (1). There is a marked increase in the rate of de novo synthesis of cholesterol in response to mitogenic stimulation (2, 3). However, the effect of mitogenic stimulation and entry into the cell cycle on the expression of LDL receptors and on the uptake of exogenous lipoprotein cholesterol has not been well defined.

Experiments from our laboratory have suggested that lymphocytes increase LDL receptor activity upon entry into the cell cycle (4). Thus, the uptake of fluorescent-labeled LDL is increased following mitogenic stimulation (4). Since uptake and utilization of lipoprotein cholesterol by proliferating lymphocytes is dependent on either apolipoprotein B or apolipoprotein E being accessible on the lipoprotein and on the function of normal LDL receptors on the lymphocyte cell surface (5), these findings suggest that the number or activity of LDL receptors may be increased by entry into the cell cycle.

The regulation of LDL receptor activity has been extensively examined in continuously cultured cells, particularly human fibroblasts (6). Most of this work has focused on the role of sterols in the regulation of LDL receptor expression. Thus, when exogenous lipoproteins are removed, there is a compensatory increase in LDL receptor activity by cells in culture, resulting from an increase in the synthesis of specific receptors (7). Recent experiments using hamster cells transfected with human LDL receptor genomic sequences fused to reporter genes indicate that negative feedback regulation of LDL receptor activity in response to sterols is mediated at the transcriptional level by sequences in the 5' flanking region of the gene (8-10). Information on the

Abbreviations: IL-2, interleukin-2; LDL, low density lipoproteins; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

regulation of LDL receptor gene expression in intact cells and the impact of influences other than ambient LDL is currently incomplete.

In previous studies, human peripheral blood mononuclear cells (PBMC) have been shown to be a useful model system to examine regulation of LDL receptor gene expression (11). Thus, following *in vitro* culture, LDL receptor mRNA levels in PBMC increased rapidly and were subject to regulation by LDL and oxygenated sterols (11). In continuously cultured nonlymphoid cells, it has been suggested that alterations in functional activity may affect LDL receptor gene expression. Evidence for this includes the finding that transcription of the LDL receptor gene was transiently increased in the early stages of phorbol ester-induced differentiation of a human monocytic leukemia cell line (12). In addition, LDL receptor mRNA levels rapidly increased in confluent fibroblasts stimulated with insulin or platelet-derived growth factor (13). These studies have raised the possibility that mitogenic signals and/or activation of tyrosine kinase or protein kinase C may increase transcription of the LDL receptor gene. Use of human PBMC provides a model system to examine this possibility with cells that are initially quiescent and in the G₀ phase of the cell cycle. Changes manifested by these cells may reflect physiologic conditions more accurately since they have not been maintained in culture for long periods of time before study. The current experiments, therefore, utilized freshly isolated PBMC to assess the effect of mitogenic stimulation on LDL receptor mRNA levels.

MATERIALS AND METHODS

Cell preparation and culture

PBMC were isolated from anticoagulated venous blood of normal adults as previously described (1-5). For some experiments, T cell-enriched populations (> 95% rosette positive) were obtained by rosetting with neuraminidase-treated sheep red blood cells followed by passing rosette-positive cells over a nylon wool column as detailed (2). Cells were cultured in RPMI 1640 medium (Inland Laboratories, Austin, TX) supplemented with 1% lipoprotein-poor plasma (d > 1.230 g/ml) as described previously (1-5). Phytohemagglutinin (PHA, Wellcome Reagents Ltd., Research Triangle Park, NC) at the previously determined optimal concentration (0.5 µg/ml) was used as the mitogenic stimulus for all experiments. Where indicated, cultures were further supplemented with 25-hydroxycholesterol (Steraloids, Inc., Wilton, NH), the sodium salt of mevalonate, lovastatin (Merck, Sharp and Dohme, Rahway, NJ), or human LDL (d 1.020-1.050 g/ml) prepared as detailed previously (4, 5). Incubations were carried out in sterile tissue culture flasks or 17 × 100mm polypropylene tubes with 1-2 × 10⁶ cells/ml initially cultured. Rates of endogenous sterol synthesis were measured as previously described (5). Incorporation

of radiolabeled mevalonate into lipid products was determined by a modification of the method of Faust, Goldstein, and Brown (14) as detailed (15). Briefly, cells were incubated with trace amounts of [³H]mevalonate (0.1 µCi/nmol) and harvested after a 24-h incubation. After extraction, lipids were separated by thin-layer chromatography and identified by iodine visualization; then chromatograms were subjected to fluorography after treatment with En³Hance[®].

Measurement of mRNA by nuclease protection

Total RNA was isolated from PBMC and T cell-enriched populations in a solution of 5 M guanidinium thiocyanate (Fluka Chemical Corporation, Ronkonkoma, NY), 30 mM sodium citrate, 20 mM β-lauroyl sarcosine, and 100 mM β-mercaptoethanol, followed by centrifugation through a 5.7 M cesium chloride (Bethesda Research Laboratories, Gaithersburg, MD) cushion as described (16). Single stranded probes for human β-actin and LDL receptor were prepared as previously described (11). Briefly, a 382 nucleotide (nt) *Sma*I-*Msp*I fragment from the coding region of the β-actin cDNA pHFβ-A1 (17, 18), encompassing nucleotides 124-505, was subcloned into the bacteriophage M13mpl9 vector and a 264 nt *Pst*I fragment from the cDNA-containing plasmid pLDLR2 (19) encoding parts of exons 8 and 9 of the human LDL receptor (20) was subcloned into the *Pst*I site of M13mpl9 vector. The interleukin 2 (IL-2) receptor α-chain clone pIL2R3 containing a cDNA insert for the human p55 (CD25, Tac antigen) α-chain of the IL-2 receptor (21), was a gift from Dr. W. C. Greene (Duke University Medical Center, Durham, NC). A 138 nt *Pst*I-*Sau*3A fragment spanning exons 1 and 2 (22) was subcloned into the *Pst*I-*Bam*HI site of the M13mpl9 vector, and a 439 nt *Pst*I-*Xba*I fragment encoding exons 4-8 (22) was subcloned in the *Pst*I-*Xba*I site of the M13mpl9 vector. The 138 nt fragment hybridizes with all IL-2 receptor mRNAs, regardless of whether they contain the differentially spliced exon 4 or not, whereas the 439 nt fragment hybridizes completely only with functional mRNAs containing exon 4 (21). No differences were noted with results obtained with either of these probes.

Single-stranded ³²P-labeled probes were synthesized with an oligonucleotide primer in the presence of 0.75 µM [α -³²P]dCTP (~ 3000 Ci/mmol, ICN Chemical and Radioisotope Division, Irvine, CA), dCTP (1.5 µM for LDL receptor, 30 µM for IL-2 receptor, and 270 µM for β-actin probes); 0.1 mM dATP, dTTP, and dGTP, and the Klenow fragment of *E. coli* DNA polymerase as detailed (23). The extended product was digested with *Hind*III (Boehringer-Mannheim Biochemicals) and the resultant ³²P-labeled probe was purified by 7 M urea-6% polyacrylamide gel electrophoresis, electroelution, and ethanol precipitation. The sizes of the probes, including M13 vector sequence, were β-actin = 457 nt, IL-2 receptor = 504 nt, or 191 nt, and LDL receptor = 337 nt.

Total RNA (10 µg) was hybridized with multiple ³²P-labeled probes simultaneously at 48 °C overnight as described (24),

and then digested with 5 units of mung bean nuclease (Bethesda Research Laboratories) in a buffer containing 50 mM sodium chloride, 10 mM sodium acetate, pH 4.6, 1 mM zinc chloride, 1 mM β -mercaptoethanol, and 0.001% Triton X-100. After precipitation with carrier salmon sperm DNA (1 μ g), samples were analyzed on 7 M urea-6% polyacrylamide gels with 32 P-labeled *Msp*I fragments of pBR322 (New England Biolabs, Beverly, MA) as size standards.

After electrophoresis, the gels were fixed and dried before being exposed to Kodak XAR-5 film for 12-24 h at room temperature with intensifying screens. After exposure, radiolabeled bands were identified, excised, and the [32 P]cDNA content was quantified by liquid scintillation spectroscopy. The [32 P]cDNA content of identically-sized bands from lanes containing no RNA was used as a measurement of nonspecific background and was subtracted from all values. The absolute cpm incorporated into each probe varied in different experiments. Consequently, the absolute amount of protected [32 P]cDNA could not be compared between experiments. However, comparisons within an experiment in which the same [32 P]cDNA probe was used could be used to determine changes in abundance of mRNA. In some experiments, results were expressed as relative LDL receptor mRNA levels calculated using the amount of actin or IL-2 receptor mRNA to correct for procedural losses. In these

studies, experimental results are expressed relative to those of control cells normalized so that they contained 100 arbitrary units of LDL receptor mRNA.

RESULTS

Mitogenic stimulation increases LDL receptor mRNA in human PBMC

When PBMC were cultured in LDL-containing medium, stimulation with the mitogenic lectin PHA resulted in cell-cycle entry as evidenced by the appearance of mRNA for the p55 α -chain of the IL-2 receptor (Tac, CD25; Fig. 1, lane 2). PHA stimulation also markedly increased LDL receptor mRNA levels (Fig. 1). In five similar experiments, LDL receptor mRNA levels in PHA-stimulated PBMC were 9.1 ± 1.9 -fold (mean \pm SEM) greater than the level in paired, unstimulated control PBMC (Fig. 1). The increase in β -actin levels was considerably less (2.9 ± 0.7 -fold). Thus, cellular activation by mitogenic stimulation induced a substantial increase in LDL receptor expression, even though LDL was present in concentrations sufficient to saturate LDL receptors (1, 5, 6).

Induction of LDL receptor mRNA levels in unstimulated PBMC is observed after incubation in lipoprotein-deficient

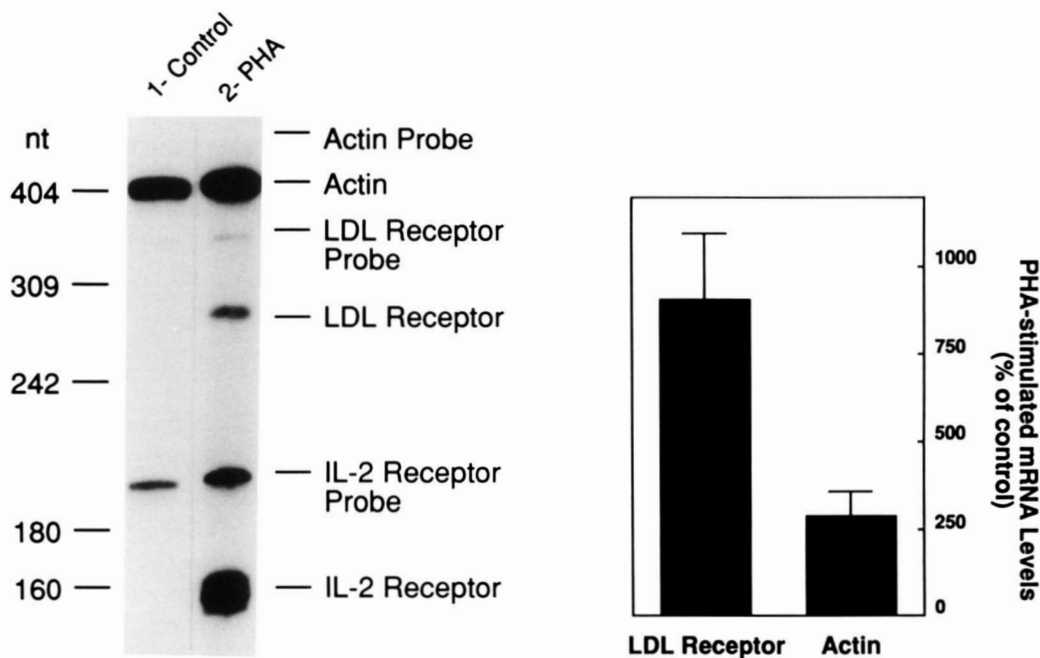


Fig. 1. Mitogenic stimulation markedly increases LDL receptor mRNA in human PBMC. PBMC were incubated for 24 h in medium containing 50 μ g LDL cholesterol/ml and with or without phytohemagglutinin (PHA). Total RNA was isolated and 10 μ g was hybridized with 32 P-labeled probes and protected probes were detected as described in Materials and Methods. The bands were identified by comparison to a 32 P-labeled *Msp*I-digest of pBR322 separated by electrophoresis in adjacent lanes (actin probe = 457 nt; actin protected band = 389 nt; LDL receptor probe = 337 nt; LDL receptor protected band = 271 nt; IL-2 receptor probe = 191 nt; IL-2 receptor protected band = 149 nt). The mRNA protected 32 P-labeled probe was quantified by liquid scintillation spectroscopy, as described in Materials and Methods. Left panel: Representative autoradiograph depicting effects of mitogenic stimulation on mRNA levels; Right panel: Quantitative results of five separate experiments measuring effects of PHA stimulation on mRNA levels, presented as percentage of control, unstimulated mRNA levels.

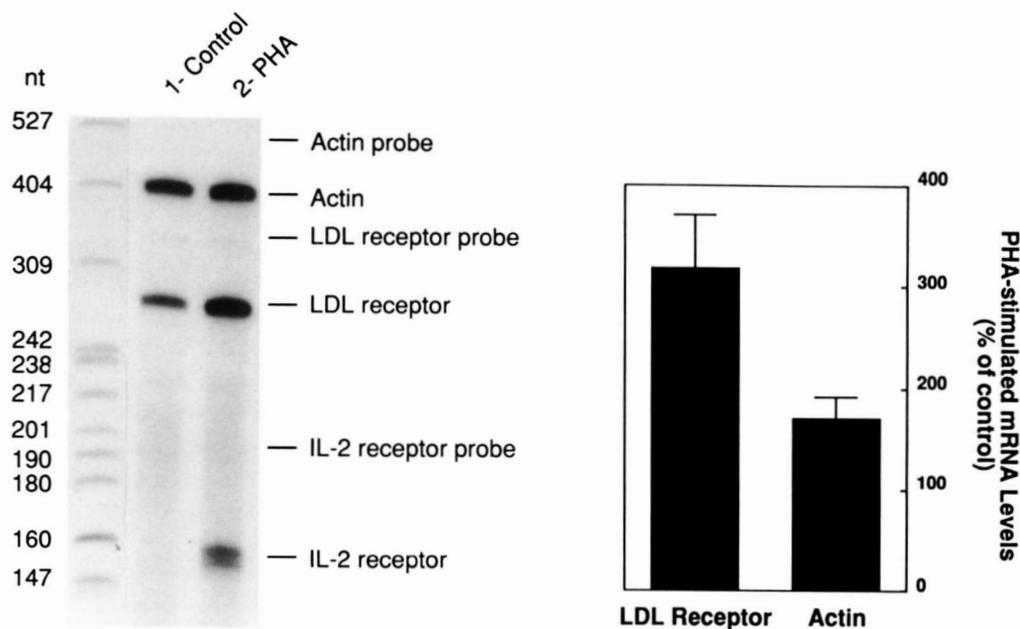


Fig. 2. Mitogenic stimulation increases LDL receptor mRNA in maximally induced human PBMC. PBMC were incubated for 24 h in lipoprotein-deficient medium with or without phytohemagglutinin (PHA). Total RNA was isolated and 10 μ g was hybridized with 32 P-labeled probes and protected probes were detected and quantitated as described in Materials and Methods. Left panel: Representative autoradiograph depicting effects of mitogenic stimulation on mRNA levels; Right panel: Quantitative results of 10 separate experiments measuring effects of PHA stimulation on mRNA levels, presented as percentage of control, unstimulated mRNA levels.

medium (11). We therefore determined whether mitogenic stimulation was able to increase LDL receptor gene expression further when PBMC were cultured in lipoprotein-depleted medium. After *in vitro* incubation for 24 h in lipoprotein-deficient medium, LDL receptor mRNA was readily detected in unstimulated PBMC (Fig. 2, lane 1). Stimulation of PBMC with PHA further increased LDL receptor mRNA levels by 3-fold above that observed in unstimulated control cells (Fig. 2, lane 2). In ten other experiments carried out in a similar manner, LDL receptor mRNA levels in PHA-stimulated PBMC cultured in lipoprotein deficient medium were 3.2 ± 0.5 -fold (mean \pm SEM) greater than the level in unstimulated PBMC (Fig. 2). For comparison, β -actin mRNA levels in PHA-stimulated PBMC were 1.7 ± 0.2 -fold that found in unstimulated cells. mRNA levels for the p55 α -chain of the IL-2 receptor were similarly increased in PHA-stimulated PBMC regardless of the presence of LDL (Fig. 1 and Fig. 2). Identical results for IL-2 receptor mRNA levels were obtained with the cDNA probe corresponding to exons 1 and 2 (149 nt protected probe, Fig. 1) that hybridizes with all IL-2 receptor mRNA species and with the probe specific for exons 4–8 (449 nt protected probe, see Fig. 9 for comparison) that is only completely protected by functional mRNA containing exon 4 (21).

The next series of experiments was carried out to confirm that changes in LDL receptor gene expression were occurring in the responding T cell population and not in other cells in PBMC. Mitogenic stimulation of T cell-enriched

populations increased LDL receptor mRNA levels by 4-fold above that noted in unstimulated T cells when cultures were carried out in lipoprotein-depleted medium (Fig. 3, lane 4). Of note, when cultures were supplemented with LDL (5 or 50 μ g cholesterol/ml), the relative effect of mitogenic stimulation was even greater, increasing LDL receptor mRNA levels by 8- to 9-fold. Thus, cell cycle entry induced by mitogenic stimulation resulted in increased LDL receptor gene expression in the responding T lymphocytes. Moreover, LDL receptor mRNA levels in PHA-stimulated T lymphocytes were always higher than in control, unstimulated cells, regardless of the presence of exogenous LDL.

Steroid regulation of LDL receptor gene expression in mitogen-stimulated lymphocytes

In unstimulated PBMC, LDL receptor mRNA levels are negatively regulated by exogenous LDL (11). Experiments were undertaken to determine whether LDL receptor gene expression was similarly regulated in PHA-stimulated PBMC. The addition of LDL (50 μ g cholesterol/ml) reduced LDL receptor mRNA levels in control PBMC by $67 \pm 3\%$ (mean \pm SEM, $n = 5$), but in PHA-stimulated PBMC LDL only decreased mRNA levels by $26 \pm 12\%$ (Fig. 4). LDL cholesterol had no effect on either actin or IL-2 receptor mRNA levels. A similar result was obtained with purified T cells, in which LDL reduced LDL receptor mRNA levels in resting cells to a greater degree than in PHA-stimulated cells (Fig. 3). Concentrations of LDL as low as 5 μ g cholesterol-

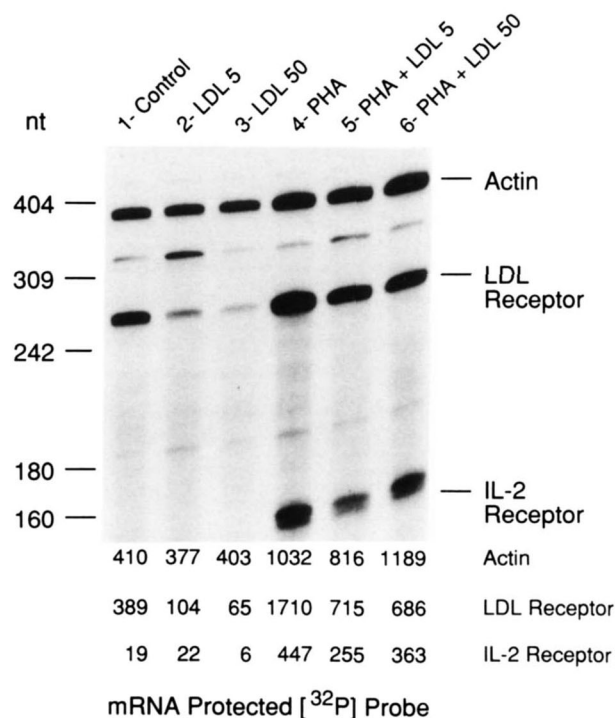


Fig. 3. PHA induces LDL receptor gene expression in human T cells. T cell-enriched populations were prepared and RNA was isolated after a 24-h incubation in lipoprotein-deficient medium with or without phytohemagglutinin (PHA) and LDL (5 or 50 μ g cholesterol/ml) as indicated. RNA (10 μ g) was hybridized with ³²P-labeled probes and actin, LDL receptor, and IL-2 receptor bands resistant to mung bean nuclease digestion were identified and quantified. Background cpm were actin = 21, LDL receptor = 66, IL-2 receptor = 139. Relative LDL receptor mRNA levels, normalized for actin mRNA in resting and PHA-stimulated control T cells, were: lane 1 = 100; lane 2 = 29; lane 3 = 16; lane 4 = 440; lane 5 = 232; lane 6 = 153.

ol/ml also down-regulated LDL receptor mRNA levels (Fig. 3, lanes 2 and 5). In unstimulated cells, such low concentrations of LDL (5 μ g cholesterol/ml) regulated LDL receptor gene expression almost as well as higher concentrations (50 μ g cholesterol/ml), achieving more than 80% of the effect (81 \pm 5%, mean \pm SEM, n = 3). In contrast, regulation by 5 μ g/ml LDL cholesterol in PHA-stimulated cells was not as effective, down-regulating LDL receptor mRNA levels only 67 \pm 5% as effectively as 50 μ g/ml LDL. The results indicated that equimolar concentrations of LDL down-regulated LDL receptor mRNA less effectively in PHA-stimulated compared to control PBMC.

The possibility that mitogenic stimulation of PBMC altered the sensitivity of other metabolic events in cellular sterol homeostasis to regulation of LDL was also examined. In these studies, the effect of LDL on endogenous sterol synthesis, measured by quantifying the rate of incorporation of radiolabeled acetate into digitonin-precipitable sterols, was determined. As shown in Fig. 4, LDL decreased endogenous sterol synthesis by 72 \pm 6% (mean \pm SEM, n = 6) in unstimulated PBMC (from 9.0 \pm 1.1 to 2.3 \pm 0.4 pmol/h

per 10⁶ cells) and by 69 \pm 3% in PHA-stimulated PBMC (from 175.9 \pm 16.0 to 52.4 \pm 1.0 pmol/h per 10⁶ cells). Therefore, LDL regulation of endogenous sterol synthesis was unlike regulation of LDL receptor gene expression in that the degree of regulation by LDL was comparable in unstimulated and PHA-stimulated PBMC.

LDL receptor gene expression was additionally examined in PBMC obtained from a patient with LDL receptor-negative homozygous familial hypercholesterolemia. This patient is a compound heterozygote, having one null allele and a second allele encoding nonfunctional mRNA. LDL failed to regulate LDL receptor mRNA levels in PHA-stimulated PBMC obtained from this patient (Fig. 5). These results support the conclusion that exogenous LDL modulated LDL receptor mRNA levels in normal lymphocytes by a mechanism dependent upon LDL receptor function.

The ability of sterols other than LDL cholesterol to regulate LDL receptor gene expression in mitogen-stimulated PBMC was also studied. The oxygenated sterol, 25-hydroxycholesterol (0.1 μ g/ml), suppressed LDL receptor gene expression in both unstimulated and PHA-stimulated PBMC to an equivalent degree, reducing LDL receptor mRNA levels by 73 \pm 5% in control cells and by 73 \pm 8% (mean \pm SEM, n = 3) in mitogen-stimulated PBMC (Fig. 6). Although there was a marked decrease in LDL receptor mRNA levels in cultures containing 25-hydroxycholesterol, even in the presence of the oxygenated sterol, PHA stimulation augmented the level (2.1 \pm 0.6-fold, mean \pm SEM, n = 3) above that in unstimulated PBMC. Mevalonate, the precursor of endogenously synthesized cholesterol, was also able to suppress LDL receptor gene expression. In four experiments, mevalonate (10 mM) suppressed LDL receptor mRNA levels in unstimulated PBMC by 30 \pm 7% (mean \pm SEM) and

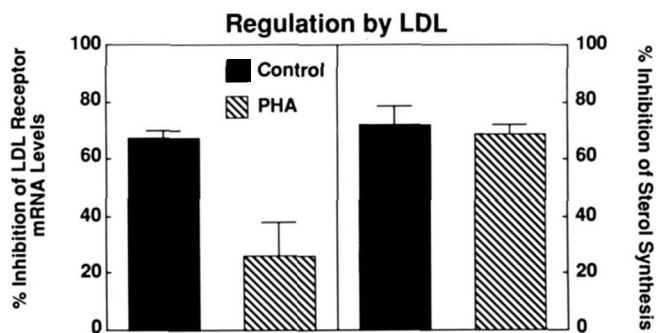


Fig. 4. Regulation of LDL receptor mRNA and endogenous sterol synthesis by LDL. PBMC were incubated in lipoprotein-deficient medium with or without phytohemagglutinin (PHA). LDL (50 μ g cholesterol/ml) was added to paired samples. After a 24-h incubation, sterol synthesis was measured or total RNA was isolated and 10 μ g was hybridized with ³²P-labeled probes; actin, LDL receptor and IL-2 receptor bands resistant to mung bean nuclease digestion were identified and quantified, and the percentage inhibition was calculated. Left panel: Regulation of LDL receptor mRNA by exogenous LDL; results are mean \pm SEM of five separate experiments. Right panel: Regulation of sterol synthesis by exogenous LDL; results are mean \pm SEM of six separate experiments.

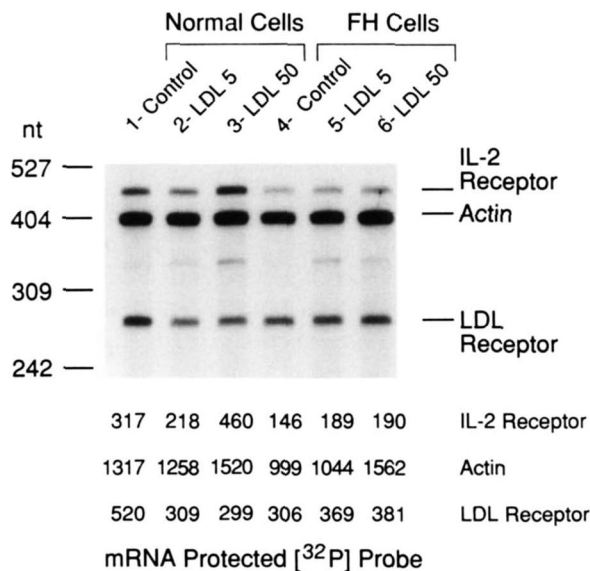


Fig. 5. Absence of LDL regulation of LDL receptor gene expression in familial hypercholesterolemia. PBMC were obtained from a normal control and from a patient with LDL receptor-negative (mRNA-positive) homozygous familial hypercholesterolemia (FH), incubated in lipoprotein-deficient medium with phytohemagglutinin (PHA) and with or without LDL in varying concentrations as indicated. Total RNA was isolated and 10 μ g was hybridized with 32 P-labeled probes and protected probes were detected and quantified as described in Materials and Methods. Background cpm were IL-2 receptor = 16, actin = 20, LDL receptor = 11. Relative amounts of LDL receptor mRNA, normalized for the amounts of IL-2 receptor mRNA were: lane 1 = 100; lane 2 = 86; lane 3 = 40; and lane 4 = 100; lane 5 = 93; lane 6 = 96.

by $23 \pm 5\%$ in paired PHA-stimulated PBMC (Fig. 6). Mevalonate had no effect on the ability of PHA to induce LDL receptor gene expression. Thus, PHA stimulation increased LDL receptor mRNA levels by 4.3 ± 0.4 -fold (mean \pm SEM, $n = 4$) in cultures incubated in lipoprotein-deficient medium and by 5 ± 1 -fold when mevalonate was added. The addition of 25-hydroxycholesterol or mevalonate had no consistent effect on β -actin mRNA. Furthermore, levels of IL-2 receptor mRNA were not substantially changed by additions of 25-hydroxycholesterol or mevalonate (data not shown).

Kinetics of induction of LDL receptor gene expression

LDL receptor gene expression is rapidly induced upon *in vitro* incubation of freshly isolated PBMC in the absence of mitogenic stimulation (11). The increase following PHA stimulation was slower (Fig. 7). Thus, PHA had no effect on LDL receptor mRNA levels measured after a 2-h incubation in lipoprotein-depleted medium (0.9 ± 0.2 -fold that in paired, unstimulated control PBMC; mean \pm SEM, $n = 3$; data not shown). PHA induced a modest increase in LDL receptor mRNA levels above that resulting from *in vitro* incubation in lipoprotein-deficient medium alone after a 4-h incubation. However, maximum levels were not attained until 20 h after PHA stimulation in most experiments (Fig.

7). When cultures were supplemented with LDL, increases were detected by 3–4 after mitogenic stimulation. Once again, however, maximum levels were not consistently achieved until after 20 h of stimulation (Fig. 7). IL-2 receptor mRNA was minimally increased after 3–4 h of culture with PHA, and continued to increase thereafter (Fig. 7). The kinetics of IL-2 receptor mRNA induction were unaffected by LDL.

Maintenance of LDL receptor mRNA levels in mitogen-stimulated human PBMC

An increase in mRNA levels can result from either increased transcription, or decreased degradation of mRNA, or both. The possibility that PHA may have increased LDL receptor mRNA levels by decreasing its degradation was, therefore, examined. After incubation for 24 h, actinomycin D was added to prevent further synthesis of mRNA, and total RNA was isolated 2 h later. During this 2-h period, the level of LDL receptor mRNA decreased by 75% in mitogen-activated PBMC (Fig. 8, lanes 3 and 4), whereas there was only a 21% decrease in actin mRNA and a 44% decrease in IL-2 receptor mRNA. In three other experiments, LDL receptor mRNA levels in PHA-stimulated PBMC decreased by $72 \pm 2\%$ (mean \pm SEM) during a 2-h incubation with actinomycin D (Fig. 8). LDL receptor mRNA levels decreased by $55 \pm 2\%$ during a 2-h incubation of parallel cultures of unstimulated PBMC with actinomycin D (Fig. 8). The calculated half-life of LDL receptor mRNA was 106 ± 6 min (mean \pm SEM, $n = 3$) in control PBMC and was considerably shorter (68 ± 5 min) in corresponding mitogen-

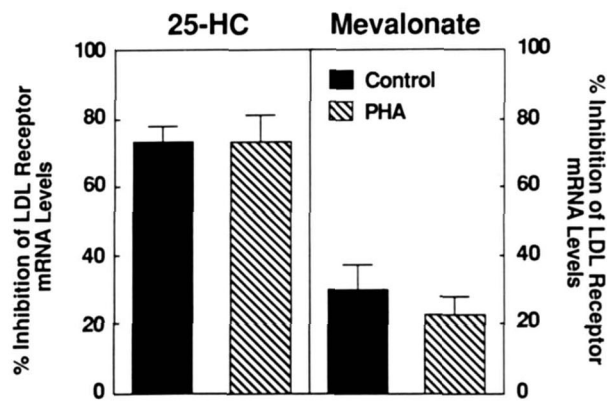


Fig. 6. Regulation of LDL receptor mRNA by oxygenated sterols and mevalonate. PBMC were incubated in lipoprotein-deficient medium with or without phytohemagglutinin (PHA). 25-Hydroxycholesterol (25-HC, 0.1 μ g/ml; left panel) or mevalonate (10 mM; right panel) was added to paired samples. After a 24-h incubation total RNA was isolated and 10 μ g was hybridized with 32 P-labeled probes; actin, LDL receptor and IL-2 receptor bands resistant to mung bean nuclease digestion were identified and quantified and the percentage inhibition was calculated. Left panel: Regulation by 25-HC, results are mean \pm SEM of three separate experiments. Right panel: Regulation by mevalonate, results are mean \pm SEM of four separate experiments. Actin and IL-2 receptor mRNA levels were unaffected by additions to either unstimulated or PHA-stimulated cells (actin mRNA level = mean 5.3% inhibition; IL-2 receptor mRNA level = mean 4.0% inhibition).

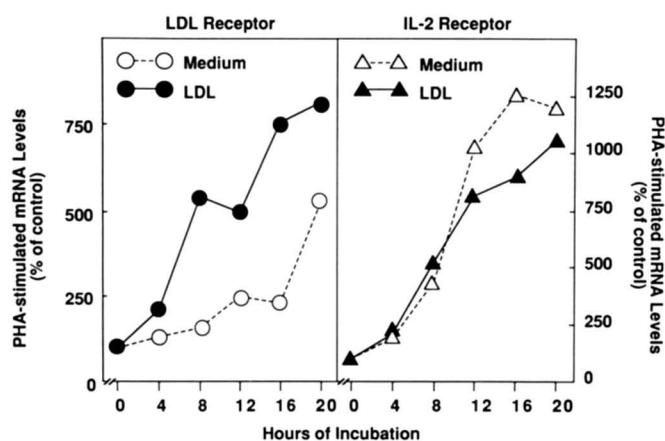


Fig. 7. Kinetics of the increase in LDL receptor mRNA levels in mitogen-stimulated PBMC. PBMC were obtained and RNA was isolated after varying lengths of incubation with or without PHA in lipoprotein-deficient medium (medium; \circ — \circ , \triangle — \triangle) or in medium supplemented with 50 μ g LDL cholesterol/ml (LDL; \bullet — \bullet , \blacktriangle — \blacktriangle). RNA (10 μ g) was hybridized with 32 P-labeled probes and actin, LDL receptor, and IL-2 receptor bands resistant to mung bean nuclease digestion were identified and quantified and the effect of mitogenic stimulation was calculated. Each point is mean result of one-five separate estimations and represents the differences in specific mRNA levels between mitogen-stimulated and control PBMC incubated in each culture condition.

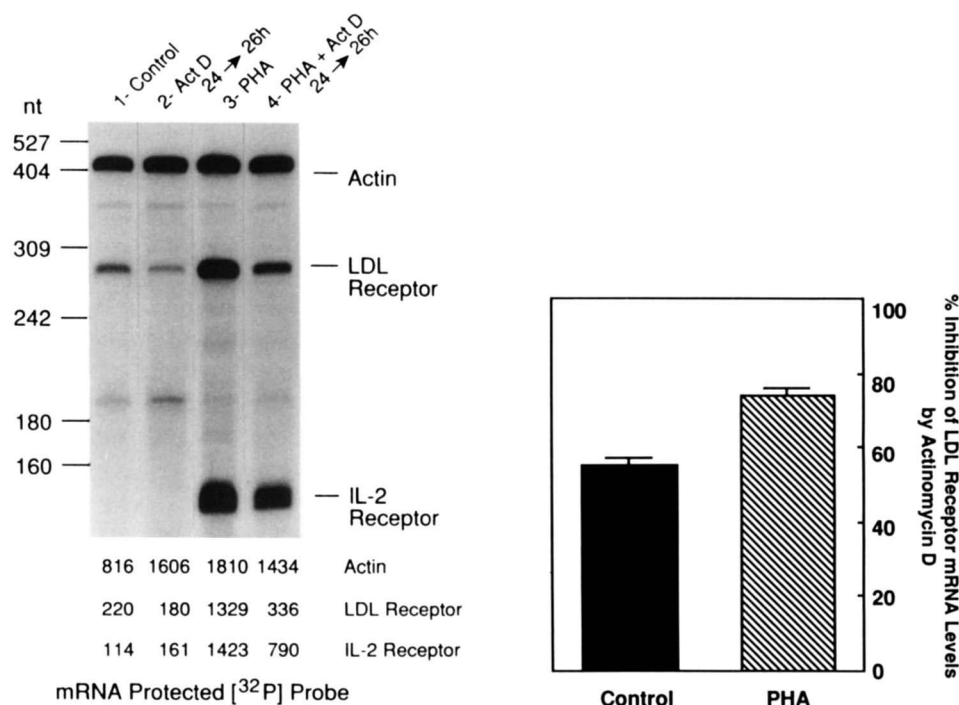


Fig. 8. Maintenance of LDL receptor mRNA levels in mitogen-stimulated PBMC requires RNA synthesis. PBMC were prepared and RNA was isolated 24 h (lanes 1 and 3) or actinomycin D (10 μ g/ml) was added after 24 h and RNA was isolated after 26 h of incubation (lanes 2 and 4) in lipoprotein-deficient medium with or without phytohemagglutinin (PHA). Total RNA (10 μ g) was hybridized with 32 P-labeled probes and actin, LDL receptor, and IL-2 receptor bands resistant to mung bean nuclease digestion were identified and quantified. Left panel: Representative autoradiograph; background cpm were: actin = 14, LDL receptor = 34, IL-2 receptor = 134. Right panel: Quantitative results of three separate experiments measuring effects of actinomycin D addition as percentage of LDL receptor mRNA level in the absence of actinomycin D.

stimulated PBMC. Additional experiments quantified LDL receptor mRNA at varying intervals after the addition of actinomycin D, and yielded identical results (data not shown). These results demonstrate that the half-life of LDL receptor mRNA had not been prolonged by mitogenic stimulation and, therefore, strongly suggest that the increase in LDL receptor mRNA observed with PHA stimulation was the result not of mRNA stabilization but rather of increased transcription.

The following experiments examined whether the increased LDL receptor gene expression associated with mitogenic stimulation was dependent on protein synthesis. When cycloheximide was present during the entire 24-h incubation, mitogen-induced increases in LDL receptor mRNA levels were completely prevented (Fig. 9, left panel, lane 4). Moreover, LDL receptor mRNA levels in control cells were decreased by 80% (Fig. 9, left panel, lane 2). Furthermore, when cycloheximide was present for only the last 4 h of the 24-h incubation, LDL receptor mRNA levels were decreased by nearly 60% in PHA-stimulated PBMC (Fig. 9, right panel, lane 4) and by 40% in control cells (Fig. 9, right panel, lane 3), falling to a level similar to that observed after the addition of actinomycin D (Fig. 9, right

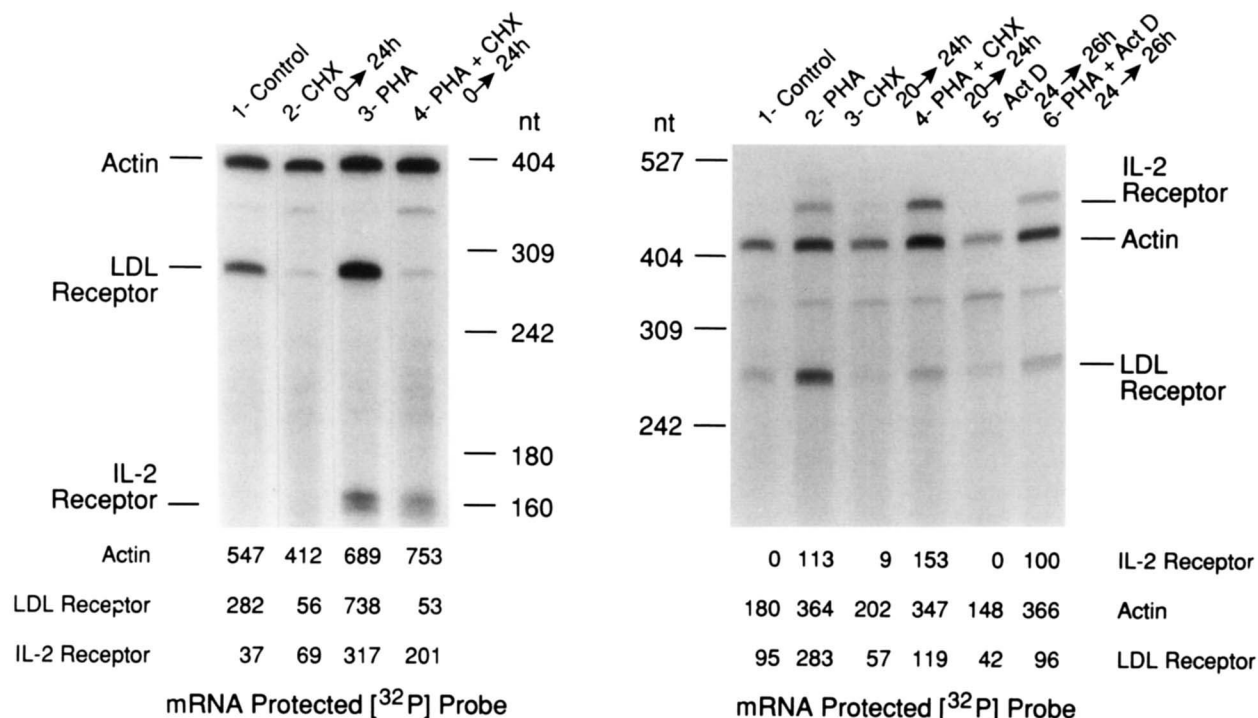


Fig. 9. LDL receptor mRNA in PBMC; requirement for protein synthesis. PBMC were isolated from 2 different individuals (experiment 1, left panel; experiment 2, right panel) and incubated for 24 h (experiment 1 and experiment 2, lanes 1-4) or 26 h (experiment 2, lanes 5 and 6) in lipoprotein-deficient medium with or without phytohemagglutinin (PHA) before RNA was isolated. Cycloheximide (10 $\mu\text{g}/\text{ml}$) was added at the initiation of culture (experiment 1, lanes 2 and 4) or after 20 h (experiment 2, lanes 3 and 4) or actinomycin D (10 $\mu\text{g}/\text{ml}$) was added at 24 h or there were no additions (experiment 1, lanes 1 and 3; experiment 2, lanes 1 and 2). RNA (10 μg) was hybridized with ^{32}P -labeled probes and actin, LDL receptor, and IL-2 receptor bands resistant to mung bean nuclease digestion were identified and quantified. Background cpm were: experiment 1: actin = 20, LDL receptor = 65, IL-2 receptor = 130; experiment 2: IL-2 receptor = 29, actin = 22, LDL receptor = 37.

panel, lanes 5 and 6). Levels of actin mRNA were largely unaffected by cycloheximide. Similarly, IL-2 receptor mRNA levels in PHA-stimulated cells were unaltered (Fig. 9) or only modestly decreased by prolonged incubation with cycloheximide (37% inhibition after 24 h). Thus, ongoing protein synthesis is required for the increase in, and maintenance of, LDL receptor mRNA observed in mitogen-stimulated PBMC.

Inhibition of endogenous sterol synthesis increases LDL receptor mRNA

The possibility that loss of cholesterol might account for the increase in LDL receptor mRNA in mitogen-stimulated PBMC was next examined. PBMC were deprived of cholesterol by culturing them in lipoprotein-deficient medium and inhibiting endogenous sterol synthesis with lovastatin for 24 h before measurement of LDL receptor mRNA levels. As seen in Fig. 10, the addition of 0.5 μM lovastatin only modestly increased LDL receptor mRNA levels. This concentration of lovastatin inhibited endogenous sterol synthesis in unstimulated control cells by $79 \pm 5\%$ (mean \pm SEM, $n = 5$) and by $77 \pm 5\%$ in corresponding PHA-stimulated PBMC (Fig. 10). A higher concentration of lovastatin (5.0 μM) suppressed sterol biosynthetic rates by $94 \pm 3\%$ (mean \pm SEM, $n = 3$) in unstimulated PBMC and by $94 \pm 2\%$ in paired

PHA-stimulated cells, but did not substantially increase LDL receptor gene expression further. Of note, lovastatin did not increase LDL receptor mRNA levels in unstimulated PBMC to the same degree as PHA stimulation. Moreover, the presence of lovastatin additionally increased LDL receptor mRNA levels in PHA-stimulated PBMC. There was a decrease in the levels of actin and IL-2 receptor mRNA in PHA-stimulated PBMC treated with lovastatin in this experiment but not in a second experiment. These results indicate that inhibition of endogenous sterol synthesis only modestly increases LDL receptor gene expression above that resulting either from *in vitro* incubation in lipoprotein-deficient medium or mitogenic stimulation, and, therefore, makes it unlikely that the increase in LDL receptor mRNA levels with mitogenic stimulation can be accounted for by deprivation of cholesterol.

To confirm that functional sterol deprivation did not account for the increase in LDL receptor mRNA observed with mitogenic stimulation, PBMC were incubated with trace quantities of radiolabeled mevalonate and lipid products were identified by thin-layer chromatography and fluorography (Fig. 11). In unstimulated PBMC, mevalonate was incorporated into ubiquinone, cholesterol, and intermediates of the sterol synthetic pathway. Of greatest importance was the

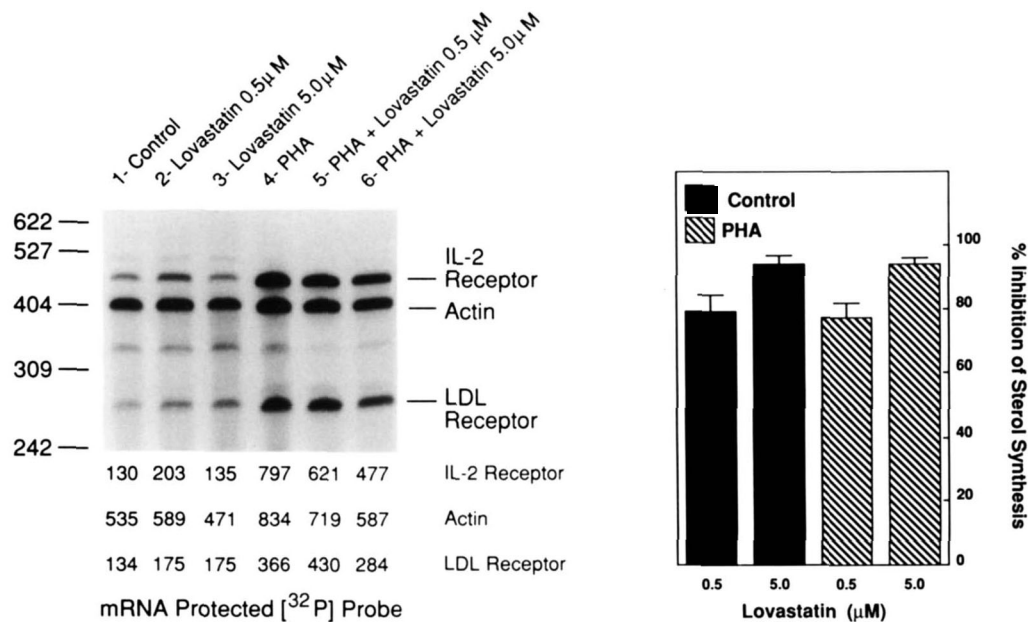


Fig. 10. Inhibition of endogenous sterol synthesis increases LDL receptor mRNA. PBMC were incubated in lipoprotein-deficient medium with or without phytohemagglutinin (PHA) and lovastatin (0.5 μM, lanes 2 and 5; 5.0 μM, lanes 3 and 6) for 24 h. Sterol synthesis was measured or total RNA was isolated and 10 μg was hybridized with ³²P-labeled probes; actin, LDL receptor, and IL-2 receptor bands resistant to mung bean nuclease digestion were identified and quantified and the effect of lovastatin was calculated. Left panel: Representative autoradiograph. Right panel: Inhibition of endogenous sterol synthesis by lovastatin, results are mean ± SEM of three (0.5 μM) or five (5.0 μM) separate experiments.

finding of radiolabeled cholesteryl ester. Cholesteryl esters were similarly synthesized by PHA-stimulated PBMC. In the presence of exogenous LDL, the relative incorporation of newly synthesized cholesterol into the ester fraction increased in both unstimulated and PHA-stimulated PBMC. Since cholesteryl esters are thought to be a storage form of cholesterol not synthesized until functional demand has been met, these results indicate that PHA-stimulated PBMC did not become cholesterol deficient, and, therefore, that the effect of mitogenic stimulation on LDL receptor gene expression could not be accounted for by sterol deprivation.

DISCUSSION

The current studies demonstrate that mitogenic stimulation of human T cells leads to an increase in LDL receptor gene expression. The mitogen-induced increase in LDL receptor mRNA levels was dependent on protein synthesis and could not be accounted for by altered mRNA stability. The increase in LDL receptor gene expression was subject to negative feedback regulation by exogenous sterols. However, mitogen-stimulated cells always contained substantially higher levels of LDL receptor mRNA than corresponding resting cells exposed to the same concentration of exogenous LDL. Indeed, when the concentration of LDL cholesterol in the medium was sufficient to saturate LDL receptors (1, 5, 6), mitogen-activated lymphocytes had 9-fold higher levels of LDL receptor mRNA than control, unstimu-

lated cells. These results indicate that mitogenic stimulation provides a signal that increases LDL receptor gene expression over and above that predicted from the concentration of sterols in the medium.

Induction of LDL receptor gene expression with mitogenic stimulation was most likely the result of increased transcription and not the result of mRNA stabilization. Thus, the half-life of LDL receptor mRNA was not lengthened by mitogenic stimulation, and, in fact, was considerably shorter in mitogen-stimulated PBMC than in resting cells. These results support the conclusion that mitogenic stimulation increased transcription of the LDL receptor gene.

LDL receptor gene expression in both mitogen-stimulated and control cells was dependent on protein synthesis. Indeed, ongoing synthesis of new protein was necessary for continued transcription of the LDL receptor gene. In contrast, cycloheximide had no consistent effect on IL-2 receptor and actin mRNA levels in mitogen-activated or unstimulated control PBMC. These latter observations indicate that the effect of cycloheximide on LDL receptor mRNA levels was not the result of decreased cell viability, a generalized decrease in transcription, or a nonspecific increase in mRNA degradation. The current studies cannot determine whether the protein(s) required for LDL receptor gene expression in mitogen-stimulated PBMC are identical to those necessary to maintain LDL receptor mRNA levels in unstimulated cells. The findings in both mitogen-activated and unstimulated control PBMC, however, are in marked contrast to

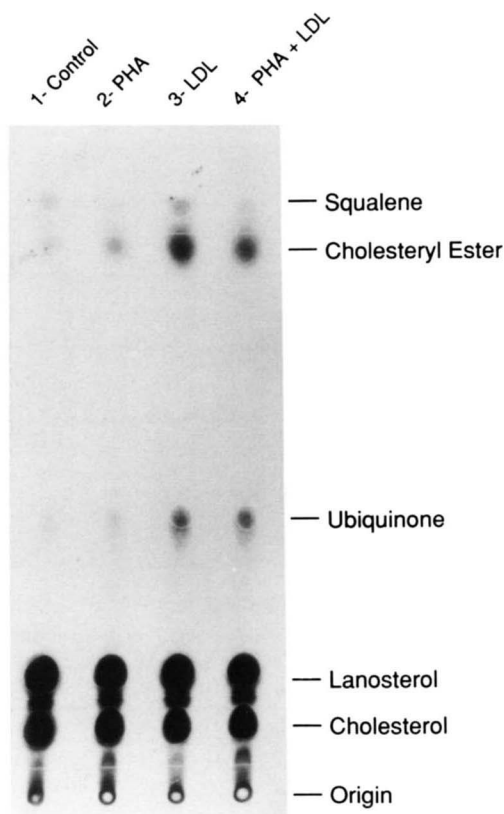


Fig. II. Synthesis of cholesteryl esters in control and PHA-stimulated PBMC. PBMC were incubated for 24 h in lipoprotein-deficient medium containing [^3H]mevalonate and with or without phytohemagglutinin (PHA) and LDL ($50\ \mu\text{g}$ cholesterol/ml) as indicated. Lipids were extracted, separated by thin-layer chromatography, identified by iodine visualization, and a fluorogram was obtained as detailed in Materials and Methods.

the effects of cycloheximide on LDL receptor mRNA levels of continuously cultured nonlymphoid cells (12, 25). Thus, inhibition of protein synthesis with cycloheximide transiently increased LDL receptor gene transcription in a human monocytic leukemia cell line and in human fibroblasts (12, 25). The putative negative regulatory protein proposed to explain these latter findings may not be expressed in PBMC. Alternatively, there may be differences in the expression of positive regulatory proteins. Regulation of LDL receptor gene expression has been shown to depend on the presence of three imperfect direct repeats in the 5' flanking region of the gene (8-10). Since all three can act in a positive manner (9), any or all may be involved in the increased transcription that results from mitogenic stimulation. Thus, mitogenic stimulation may increase either the concentration or effect of a putative positive transcription factor, or both, and thereby account for the current findings.

Another possible explanation is that mitogenic stimulation decreased the effectiveness or availability of a negatively-acting transcription factor, such as a sterol-dependent repressor protein. LDL, in concentrations that saturate LDL receptors,

down-regulated LDL receptor gene expression in PBMC by decreasing gene transcription, since the half-life of LDL receptor mRNA in resting and mitogen-stimulated PBMC was not altered by LDL (data not shown). Furthermore, the effect of LDL was specific since IL-2 receptor and actin mRNA levels were not altered by LDL. However, down-regulation by LDL was substantially less in PHA-stimulated lymphocytes than in unstimulated, control PBMC. In proliferating fibroblasts, negative feedback regulation by exogenous LDL is as effective as in unstimulated PBMC, with LDL receptor mRNA levels decreasing by $77 \pm 6\%$ (mean \pm SEM, $n = 6$) after the addition of $50\ \mu\text{g}$ LDL cholesterol/ml (J. A. Cuthbert and P. E. Lipsky, unpublished data). This latter observation suggests that differences in the effectiveness of LDL regulation of LDL receptor gene expression between resting and mitogen-activated T cells cannot simply be accounted for by changes related to cell cycling per se. Taken together, these observations demonstrate that negative feedback regulation of LDL receptor mRNA levels by lipoprotein cholesterol in mitogen-stimulated lymphocytes is similar to but less efficient than in resting cells. Of note, there was no such discrepancy in negative feedback regulation of endogenous sterol synthesis. Thus, the degree of down-regulation of sterol biosynthesis by LDL in unstimulated and PHA-stimulated PBMC was comparable. This finding makes it unlikely that differences in regulatory pools of sterol can account for these results, and, in addition, suggests that 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and LDL receptor expression are regulated differently in human PBMC.

In contrast to the findings with LDL, the oxygenated sterol 25-hydroxycholesterol suppressed LDL receptor mRNA levels in both mitogen-stimulated and unstimulated PBMC to a similar degree, and far more effectively than LDL itself. Although 25-hydroxycholesterol down-regulated LDL receptor gene expression more effectively than native LDL, PHA was still able to increase LDL receptor mRNA levels compared to that noted in control cells cultured with 25-hydroxycholesterol. Recent studies suggest that, unlike oxygenated sterols, further metabolism of LDL cholesterol is required for regulatory activity (26). Mitogenic stimulation with resultant cellular activation may alter the metabolic pathway that leads to negative feedback regulation of LDL receptor gene expression by LDL-derived cholesterol. Another possibility is that oxygenated sterols may be more effective since their transport into the cell is unlikely to be altered by a decrease in LDL receptor activity. Alternatively, the regulatory elements responsible for modulation of gene expression may show an increased susceptibility to the effects of oxygenated sterols. Normal human plasma contains up to $0.25\ \mu\text{g}/\text{ml}$ of the oxygenated sterol, 26-hydroxycholesterol (27), which is similar to 25-hydroxycholesterol in its capacity to regulate LDL receptor mRNA levels (data not shown). Since the majority of 26-hydroxycholesterol in plasma is associated with LDL, it is possible

that the regulation of LDL receptor gene expression by LDL may be the result of oxygenated sterols present in the lipoprotein. Whether the concentration of 26-hydroxycholesterol in LDL is sufficient to account for its ability to regulate LDL receptor gene expression remains to be determined.

The exact molecular mechanism whereby mitogenic stimulation increases LDL receptor gene transcription remains unknown. A number of possibilities are immediately apparent. One potential mechanism that has to be considered is that the increase in LDL receptor mRNA levels is secondary to decreased intracellular levels of cholesterol or other sterols. In continuously cultured fibroblasts, growth factor stimulation has been shown to alter a number of sterol metabolic pathways that may affect putative regulatory pools of cholesterol (28–30). Consequently, LDL receptor mRNA levels may increase in mitogen-stimulated cells secondary to a change in a regulatory pool of cholesterol, resulting from an increased need for cholesterol for membrane synthesis. The current studies make it unlikely that a decrease in a regulatory pool of cholesterol secondary to increased demand accounts for the findings, since maximal sterol deprivation, achieved by incubation in lipoprotein-deficient medium and prevention of endogenous sterol synthesis, did not maximally induce LDL receptor gene expression. Furthermore, the increase in LDL receptor gene transcription with mitogenic stimulation occurs not only in the presence of LDL, but also before there is a need for cholesterol for membrane synthesis (1, 2). Additionally, the finding of newly synthesized cholesterol in cholesteryl esters clearly established that sterol deprivation was not occurring, since only in the presence of excess cellular cholesterol is there diversion to the storage form (6).

The degree of regulation of sterol biosynthesis by LDL was identical in unstimulated and PHA-stimulated PBMC, implying that endogenous regulatory sterol pools are comparable in these cell populations. Additional experiments, not reported here in detail, confirmed that LDL-mediated regulation of the rate-limiting enzymes in sterol synthesis was comparable in control and PHA-stimulated PBMC, whereas regulation of LDL receptor mRNA was less effective in PHA-stimulated cells. In these studies, mRNAs for the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase, and for the preceding enzyme 3-hydroxy-3-methylglutaryl coenzyme A synthase, were down-regulated in a manner similar to LDL receptor mRNA in unstimulated cells (83% inhibition of LDL receptor mRNA by LDL cholesterol, 85% inhibition of reductase mRNA, and 83% inhibition of synthase mRNA, measured on the same sample). In contrast to regulation of LDL receptor mRNA, both reductase and synthase mRNAs were also effectively down-regulated by LDL in PHA-stimulated cells (97% and 95% inhibition, respectively, for reductase and synthase mRNA compared with 67% inhibition of LDL receptor mRNA by LDL cholesterol). Insofar as analysis of these mRNA species indicates the availability of sterol regulatory pools,

it appears unlikely that the decreased regulation of LDL receptor mRNA in PHA-stimulated cells reflects a functional alteration of such putative pools. Thus, neither sterol deprivation nor alterations in particular endogenous regulatory pools of sterols appears to explain the results. It is more likely that signal transduction associated with mitogenic stimulation may directly enhance LDL receptor gene transcription together with that of other genes. For example, stimuli that activate protein kinases, especially protein kinase C are linked with increased LDL receptor gene transcription in fibroblasts and a monocytic cell line (12, 13). Mitogenic stimulation of lymphocytes is also associated with protein kinase C activation (reviewed in 31), that may, in turn, be directly responsible for the increased LDL receptor mRNA levels. In preliminary experiments, the addition of phorbol myristate acetate, to activate protein kinase C, increased LDL receptor gene expression in T lymphocytes, supporting this hypothesis.

In summary, the present findings indicate that mitogenic stimulation results in higher steady-state levels of LDL receptor mRNA, regardless of the exogenous LDL concentration. Indeed, the effect of cell cycle entry on LDL receptor gene expression is more apparent in the presence of LDL. Thus, cellular activation provides a positive signal to LDL receptor expression that increases the rate of gene transcription, even in the presence of exogenous LDL. These results, therefore, indicate that mitogenic signals as well as ambient LDL concentration together determine the level of LDL receptor gene expression. ■

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