# Transcriptional and post-transcriptional regulation of LDL receptor gene expression in PMA-treated THP-1 cells by LDL-containing immune complexes

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**Abstract** We have previously shown that uptake of low density lipoprotein-containing immune complexes (LDL-IC) by human monocyte-derived macrophages led to the transformation of these cells into foam cells and induced a paradoxical increase in receptor-mediated binding of $^{125}$I-labeled LDL due to an increase in the number of LDL receptors (LDL-R). The same metabolic changes are also observed in PMA-treated THP-1 cells after incubation for 2 h with 150 μg/ml of immune complexes containing either native, oxidized (ox), or malondialdehyde (mda) LDL. After stimulation, PMA-treated THP-1 cells showed not only a 40-fold increase in $^{125}$I-labeled LDL binding but also a 40-fold increase in the immunoreactive LDL-R protein, confirming that the increase in LDL binding is due to an increase LDL-R number. In this study we have investigated, in PMA-treated THP-1 cells, the regulatory mechanism(s) responsible for the increased receptor-mediated binding of LDL induced by LDL-IC. By Northern blot and nuclear run-on analysis we have shown transcriptional activation of the LDL-R gene with a 7-fold increase in the LDL-R mRNA level in LDL-IC stimulated cells. Due to the marked difference between the increase in LDL-R mRNA and LDL-R protein, we estimated LDL-R mRNA stability using a inhibitor chase method and have shown that LDL-IC did not alter the LDL-R mRNA stability in THP-1 cells. We have also demonstrated, using cycloheximide as a inhibitor of protein synthesis, that the marked increase in LDL-R protein observed in LDL-IC-stimulated THP-1 cells resulted from de novo synthesis of LDL-R protein. To determine whether the increase in transcriptional activity of the LDL-R gene was secondary to changes in the cholesterol regulatory pool we performed experiments in which the cell cholesterol content was modified by the addition of either 25-hydroxycholesterol and mevalonate or inhibitors of ACAT activity (SA-58035 and progesterone). These experiments showed that the enhanced LDL-R expression was not affected by the addition of any of the above compounds. In conclusion, LDL-IC induced both transcriptional and post-transcriptional activation of the LDL-R gene in PMA-treated THP-1 cells and this induction was independent of the free cholesterol content of these cells.—**Huang, Y., M. J. Ghosh, and M. F. Lopes-Virella.** Transcriptional and post-transcriptional regulation of LDL receptor gene expression in PMA-treated THP-1 cells by LDL-containing immune complexes. *J. Lipid Res.* 1997. **38:** 110–120.

**Supplementary key words** cholesterol • macrophage • Fcγ receptor

Subendothelial accumulation of cholesteryl ester (CE)-laden macrophages (foam cells) in the vessel wall is a prominent feature of early atherosclerotic lesions (1). Several mechanisms are responsible for the transformation of macrophages into foam cells. One of the mechanisms that has been well described by our laboratory (2, 3) and others (4, 5) involves the uptake of LDL-containing immune complexes (LDL-IC) through Fcγ receptors. The in vivo relevance of this mechanism has been demonstrated by studies from several independent investigators describing the presence of autoantibodies against LDL as well as LDL-IC in patients with coronary artery disease (6, 7), peripheral vascular disease (8), and in human atherosclerotic lesions (9). Studies showing a statistically significant correlation between the rate of progression of carotid atherosclerosis and the titer of autoantibodies to oxidized LDL (10) and the presence of high antibody titers against oxidatively modified LDL in patients with severe carotid atherosclerosis (11) further support the role of autoimmunity in the development and/or progression of arteriosclerosis.

Besides leading to the transformation of macrophages into foam cells, LDL-IC induce the release of biologically active mediators such as TNFα and IL-1β by macrophages (12) and leads to a paradoxical increase in LDL binding by these cells due to a marked increase

Abbreviations: CE, cholesteryl esters; LDL, low density lipoprotein; ox-LDL, copper-oxidized LDL; mdaLDL, malondialdehyde LDL; LDL-IC, LDL-containing immune complexes; LDL-R, LDL receptor; PMA, phorbol 12-myristate 13-acetate; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACAT, acyl-CoA:cholesterol acyltransferase; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein; FcγR, Fc receptor for IgG; CPP32, cysteine protease protein (molecular mass, 32 kDa).

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in LDL-R number (3). The increase in LDL-R number, in contrast to the release of cytokines, seems to be specifically induced by immune complexes containing native LDL, oxidized LDL, and malondialdehyde LDL, and not by other types of immune complexes (3) and it is dependent on the uptake of the LDL-IC by Fcγ R.

Therefore, we decided to investigate the regulatory mechanisms responsible for the increase in LDL-R expression observed in human macrophages after stimulation with LDL-IC. The large number of human peripheral monocytes necessary to perform these experiments was a limiting factor that severely curtailed our ability to perform these studies, thus we started by establishing that the human monocyte cell line THP-1 treated with PMA was a good model for human monocyte-derived macrophages. Using this cell line we have shown that both transcriptional and post-transcriptional regulation are responsible for the increased LDL-R expression. Furthermore, we have concluded that the increase in LDL-R expression observed is independent of the cell free cholesterol content.

## MATERIAL AND METHODS

### Cell culture

The human monocyte cell line THP-1 was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The cells were cultured in a supplemented Iscove modified Dulbecco's medium (IMDM) previously defined (13) to which 10% fetal calf serum was added. Transformation of the cells into macrophage-like cells was induced by treatment with 0.16 μM phorbol myristate acetate (PMA) at 37°C, for 24 h (14).

### Lipoprotein isolation, modification and radiolabeling

LDL (d 1.019–1.063 g/ml) was separated from the plasma of normal volunteers by sequential ultracentrifugation at 60,000 rpm for 24 h at 10°C in a 60-Ti rotor (Beckman, Palo Alto, CA). The isolated LDL was washed and concentrated by ultracentrifugation in a SW 41 rotor (Beckman) spun at 40,000 rpm for 24 h at 10°C. Afterwards, the LDL was dialyzed against a 0.16 M NaCl solution containing 300 μmol/L of EDTA, pH 7.4, sterilized by passage through a 0.45-μm filter (Gelman Sciences, Ann Arbor, MI) and stored under $N_2$ at 4°C.

Copper-induced oxidation of LDL was performed as we previously described (12). Briefly, isolated LDL diluted in Ham's F-10 medium was incubated with 10 mM $Cu^{2+}$ at 37°C for 24 h. The reaction was stopped by the addition of 200 mM EDTA and 40 mM butylhydroxytoluene (BHT) and the oxLDL dialyzed against PBS containing 200 mM of EDTA and 40 mM of BHT. MDA modification of LDL was performed as described by Haberland et al. (15). Briefly, LDL diluted in PBS, pH 7.4, to a concentration of 1 mg/ml was incubated with a MDA solution (0.2 mol/L in 0.1 mol/L sodium phosphate buffer, pH 6.4) for 3 h at 37°C under nitrogen. The reaction was stopped by dialysis against $Ca^{2+}$-free Tyrode's solution at 4°C.

For the binding experiments LDL was labeled with $^{125}I$ by the McFarlane procedure as modified by Bratzler et al. (16). The labeling conditions were adjusted to obtain a specific activity of 100–400 cpm/ng of protein. As previously shown (17) radioactivity of the lipid moiety of LDL in our labeling conditions constitutes less than 4% of the total radioactivity.

### Preparation of LDL-IC

LDL-IC were prepared as previously described (13). Briefly, insoluble LDL-IC were prepared by incubating overnight, at 4°C, LDL (100 μg/ml) and a rabbit anti-LDL antiserum (500 μg/ml) raised in our laboratory. These concentrations were determined by a precipitin curve that was prepared, as previously described (13), by incubating 500-μg aliquots of the antiserum with varying amounts of LDL. The antigen to antibody ratio yielding the highest amount of precipitate was considered to correspond to the equivalence point and was used for the preparation of the insoluble immune complexes. The protein content of the LDL-IC was determined by the protein assay of Lowry et al. (17) after washing the immune complexes 3 times with phosphate-buffered saline, pH 7.4. The Lowry assay was calibrated with serial dilutions of a preparation of heavily aggregated IgG with known amounts of IgG. Before addition to the cells, the LDL-IC preparations were sterilized by gamma irradiation.

### Measurement of free and, esterified cholesterol content in PMA-treated THP-1 cells

To perform cholesterol mass studies, the THP-1 cell monolayers, after being extensively washed with PBS, were extracted with isopropanol–hexane 2:3 (vol/vol) as previously described (13). Free and total cholesterol were assayed on a gas chromatograph equipped with an $H_2$ flame ionization detector. A fused silica capillary column (0.53 mm diameter, 15 m long) packed with DB17, 1 micron of thickness, was used for the chromatographic separation. The oven was maintained at 255°C during the separation. Helium was used as the gas carrier. For assay of total cholesterol the cellular extracts were evaporated to dryness and the residue was hydrolyzed by the Ishikawa method as previously described (13). Cholesteryl ester levels were obtained by subtracting free cholesterol from total cholesterol levels, β-Stigmasterol was used as an internal standard.



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### LDL binding studies

After incubation of the $^{125}$I-labeled LDL with the cells, the medium was removed and the cells were washed 2 times with phosphate-buffered saline (PBS) containing 0.2% (wt/vol) bovine serum albumin followed by two additional washes with PBS without albumin. The cells were dissolved in 0.2 M NaOH containing 1% sodium dodecyl sulfate. An aliquot of the solubilized cells was taken to determine the amount of $^{125}$I-labeled LDL radioactivity present in the cell pellet. Another aliquot was used to determine the amount of cell protein by the Lowry method (18). LDL receptor-mediated binding was calculated as the difference of the levels measured in the presence and absence of a 25-fold excess of unlabeled LDL. The results were expressed as ng of LDL bound/mg cell protein.

### Immunoblotting of LDL receptor protein

To perform immunoblotting, the THP-1 cell monolayers, after being extensively washed with PBS, were lysed with buffer containing 10 mM HEPES (pH 7.4), 200 mM NaCl, 2 mM $CaCl_2$ 2.5 mM $MgCl_2$, 2 mM PMSF, and 2% Triton X-100. The protein content of the lysate was determined by the Lowry assay. One hundred μg of protein was electrophoresed on 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (19). The nitrocellulose membrane was incubated for 1 h at room temperature with a buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, 80 mM NaCl, 5% Carnation nonfat dry milk, 0.2% NP-40, and 0.01% Antifoam A. The membrane was then incubated for 2 h at room temperature with the same buffer containing a 1:200 dilution of a rabbit antiserum against the carboxyl terminal peptide of the human LDL-R, which was kindly provided to us by Dr. Innerarity (Gladstone Foundation Laboratory for Cardiovascular Disease, University of California, at San Francisco) (20). After the incubation, the membrane was washed three times with the same buffer and then incubated for 2 h at room temperature with the same buffer containing $^{125}$I-labeled anti-rabbit IgG (specific activity of $1 \times 10^6$ cpm/ml). The nitrocellulose membrane was washed as described above and the immunoreactive protein was visualized by exposure for 18 h at −70°C to Kodak XAR-2 films. The relative concentration of LDL-R protein was estimated by scanning densitometry of the autoradiograms.

### Preparation of cDNA probes

A human 4.5 kb cDNA, encoding the entire human LDL-R sequence, was obtained from ATCC as an insert in the vector pcDV1. A 1.8 kb fragment of LDL-R cDNA was excised and purified by a Geneclean Kit (Bio 101 Inc., La Jolla, CA). The human 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase cDNA and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were also purchased from ATCC. The inserts were excised and purified as above. The cDNAs were radiolabeled using the random priming method (19).

### Northern blot and slot-blot analysis

Total cellular RNA from LDL-IC-stimulated and unstimulated PMA-treated THP-1 cells were isolated with a RNAzol kit according to the procedures from the manufacturer (Biotecx Laboratories, INC. Houston, TX). For Northern blot analysis, 10 μg of total RNA for each sample was electrophoresed in 1% agarose gel with buffer containing 20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, and 0.23 M formaldehyde. The presence of 28S and 18S RNA in a ratio of approximately 2:1 was consistently observed. The RNA on the agarose gel was transferred to a nylon membrane in 20× saline–sodium citrate (SSC, 150 mmol/L NaCl and 10 mmol/L sodium citrate). The nylon membrane was prehybridized at 42°C for 3 h in buffer containing 50% formamide and then hybridized with $^{32}$P-labeled LDL-R, HMG-CoA reductase, and GAPDH cDNAs. The hybridization was carried out for 18 h at 42°C in the presence of 50% formamide, 5× SSC, 5× Denhardt's solution, 0.1% SDS, and 2 mg/ml of denatured salmon sperm DNA. After hybridization, the nylon membrane was washed for 2 h at 60°C in a buffer containing 0.1 × SSC and 0.1% SDS. Positive hybridization was identified by exposure to Kodak XAR-2 films at −70°C. The relative concentration of LDL-R and HMG-CoA reductase mRNAs was estimated by scanning densitometry of the autoradiograms and compared to that of GAPDH mRNA. For slot blot analysis, $^{32}$P-labeled LDL-R and GAPDH cDNAs were hybridized to the RNA immobilized on the nylon membrane. The relative LDL-R mRNA concentration was determined as described above for Northern blot.

### Nuclear run-on assay

Nuclear run-on transcription assay was performed as described previously (21). After incubation with LDL-IC, the THP-1 cells were washed exhaustively with PBS and gently dislodged from the plastic surface by scraping with a rubber policeman. The cells were centrifuged and lysed in NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 5% NP-40). The nuclei were spun down at 500 *g* at 4°C and the supernatant was removed. The nuclei were resuspended and centrifuged again and the nucleus pellet was resuspended in a buffer containing 50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA, 0.125 mM PMSF and stored at −70°C. After isolation, the nuclei were counted on a hemocytometer and $10 \times 10^6$ nuclei from each treatment group were used to perform



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the transcription assay. The nuclei ($10 \times 10^6$) were suspended in buffer containing 100 mM Tris-HCl, pH 7.8, 60 mM NaCl, 50 mM ammonium sulfate, 4 mM $MgCl_2$ 1.2 mM dithiothreitol, 0.4 mM EDTA, 0.1 mM PMSF, and 30% glycerol and transcription was performed, at 37°C for 30 min, in the presence of 1 mM of each of [$^{32}P$] UTP (3000 Ci/mmol), ATP, GTP, and CTP. After the 30-min incubation, 500 μl of a solution containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM KCl, 1 mM EDTA, 0.5% SDS, 100 μg/ml proteinase K was added to the transcription mixture and the incubation was continued for an additional 30 min at 37°C. $^{32}P$-labeled RNA was isolated using RNAzol and dissolved in diethyl pyrocarbonate-treated water.

Five μg of the plasmid with the inserts of LDL-R, HMG-CoA reductase, and GAPDH cDNA were boiled in 0.1 N NaOH for 2 min, chilled quickly on ice, and then mixed with 2 M ammonium acetate. The single-stranded DNA obtained was applied to a nitrocellulose membrane mounted in a slot-blot apparatus (Bio-Rad) and used for hybridization with the radiolabeled RNA obtained previously from the nuclear run-on transcription assays. The hybridization was performed at 45°C, for 52 h in 2 ml of a buffer containing 50 mM PIPES, 0.5 M NaCl, 33% formamide, 0.1% SDS, 2 mM EDTA, and 5 mg/ml salmon sperm DNA. The nylon membranes were rinsed with 1 × SSC with 0.1% SDS once at room temperature and twice at 50°C. After drying, the nylon membranes were exposed to X-ray films and the autoradiograms were subjected to scanning densitometry.

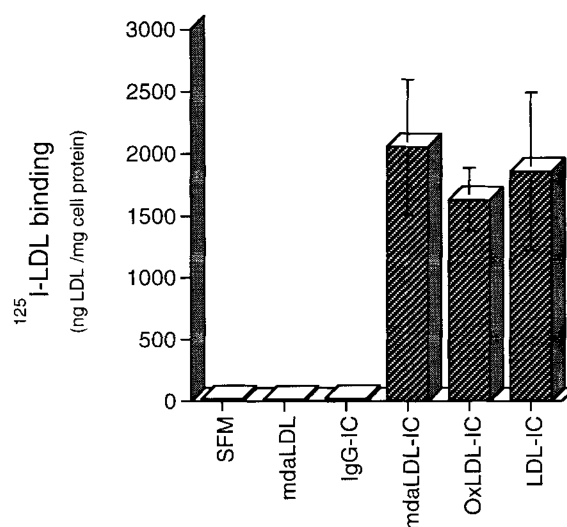


**Fig. 1.** Receptor-mediated binding of $^{125}$I-labeled LDL to the PMA-treated THP-1 cells. PMA-treated THP-1 cells were incubated, for 2 h at 37°C, with serum-free medium (SFM) alone or SFM containing 150 μg/ml of LDL, IgG-IC, LDL-IC, oxLDL-IC, or mdaLDL-IC. After the incubation, the cells were washed extensively with PBS, pH 7.4, and incubated for 4 h at 4°C with 10 μg/ml of $^{125}$I-labeled LDL in the presence or absence of a 25-fold excess of unlabeled LDL (250 μg/ml). The cells were washed with PBS, pH 7.4, as described in Methods and dissolved in 1 ml of 0.2 M NaOH containing 1% SDS. Cell-associated radioactivity was determined in a gamma counter and cell protein was determined by the Lowry method. Receptor-mediated binding was calculated as the difference between $^{125}$I-labeled LDL bound to the PMA-treated THP-1 cells in the presence and absence of unlabeled LDL. Data shown are receptor-mediated binding and represent the mean±SEM of three experiments.

## RESULTS

Several experiments were performed to establish that PMA-treated THP-1 cells were an adequate model to study the effects of LDL-IC, oxLDL-IC, or mdaLDL-IC in the regulation of LDL-R expression.

Our data showed that PMA-treated THP-1 cells, like human monocyte-derived macrophages, had a dramatic increase in LDL binding (**Fig. 1**) and CE accumulation (**Fig. 2**) after incubation with 150 μg/ml of LDL-IC, oxLDL-IC, or mdaLDL-IC. A marked increase in unesterified cholesterol was also observed in these cells as shown in **Table 1.** As the effects of LDL-IC, oxLDL-IC, and mdaLDL-IC on LDL binding to THP-1 cells were similar, LDL-IC were used in the following experiments. Time course (**Fig. 3A**) and dose–response curves (Fig. 3B) were performed to determine the time of incubation and concentration of LDL-IC needed to obtain maximal LDL binding to the cells and the time and concentration deemed to be ideal (2 h and 150 μg/ml, respectively) was used in the remaining experiments. To determine whether, as in human macrophages, the increase in LDL binding to LDL-IC-stimulated PMA-treated THP-1 cells was due to an increase in LDL-R number, not in the binding affinity of LDL to the receptor, we measured LDL-R protein by Western blot, using a rabbit antibody that recognized the LDL-R carboxyl terminal peptide. As shown in **Fig. 4,** PMA-treated THP-1 cells cultured with medium alone expressed trace amounts of LDL-R protein, while the cells stimulated with LDL-IC had approximately a 40-fold increase in LDL-R protein.

The above set of experiments confirmed that the metabolic behavior of THP-1 cells when incubated with LDL-IC mimicked that of human monocyte-derived macrophages (13) and therefore these cells are a good model for the following set of experiments that propose to investigate the regulatory mechanisms responsible for the increase in LDL binding and LDL-R protein observed after stimulation of the cells with LDL-IC. Due to the marked increase in LDL-R protein in LDL-IC-stimulated cells, it was likely that the increase was due to enhanced transcription and/or translation of the LDL-R gene. We started by assaying, by Northern blot, whether LDL-R mRNA levels in the LDL-IC-stimulated cells were increased. As it can be seen in **Fig. 5A,** a 7-



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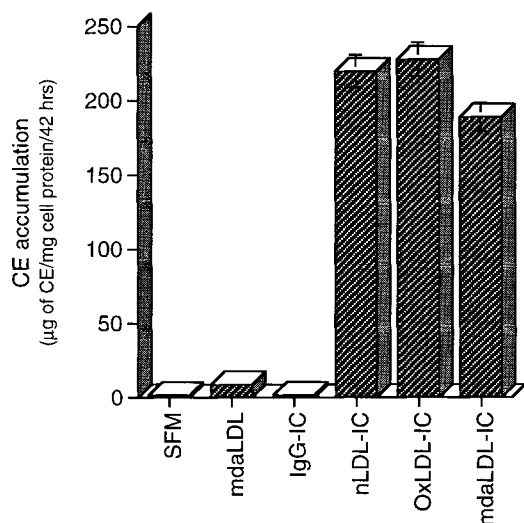


**Fig. 2.** Cholesteryl ester accumulation in PMA-treated THP-1 cells. PMA-treated THP-1 cells were incubated for 42 h, at 37°C, with serum-free medium (SFM) or with SFM containing 150 μg/ml of LDL, IgG-IC, LDL-IC, oxLDL-IC, or mdaLDL-IC. Afterwards, cell lipids were extracted using a mixture of isopropanol–hexane and free and total cholesterol were measured in the lipid extracts by gas chromatography as described in Methods. β-Stigmasterol was used as an internal standard. Cholesteryl esters were calculated by subtracting free from total cholesterol levels. The results represent the mean ± SEM of three experiments, run in triplicate.

fold increase in the LDL-R mRNA level was observed in the cells stimulated with LDL-IC for 2 h (Fig. 5A and **Table 2**). This increased steady state LDL-R mRNA level was continuously observed for 8 h (Fig. 5B). Similar experiments were also performed to determine the level of HMG-CoA reductase mRNA in unstimulated and LDL-IC stimulated cells. Our data clearly shows that no significant increase in HMG-CoA reductase mRNA is observed in THP-1 cells after stimulation with LDL-IC (Table 2).

To determine whether the increase in LDL-R mRNA

TABLE 1. Cholesteryl ester and unesterified cholesterol content in PMA-treated THP-1 cells

| Cells Incubated with | Cholesteryl Esters | Unesterified Cholesterol |
|---|---|---|
| | *μg/mg cell protein/42 h* | |
| SFM | 2 ± 1 | 24 ± 1 |
| IgG-IC | 2 ± 1 | 17 ± 1 |
| LDL-IC | 220 ± 3 | 127 ± 2 |
| oxLDL-IC | 228 ± 2 | 136 ± 3 |
| mdaLDL-IC | 189 ± 5 | 125 ± 3 |

PMA-treated THP-1 cells were incubated with serum-free medium (SFM) alone or SFM containing 150 μg/ml of IgG-IC, LDL-IC, oxLDL-IC, or mdaLDL-IC at 37°C for 42 h. Cell lipids were extracted with isopropanol–hexane and unesterified and total cholesterol were assayed by gas chromatography as described in Methods. Cholesteryl esters were calculated by subtracting unesterified cholesterol levels from total cholesterol levels. The data are representative of three experiments with similar results.

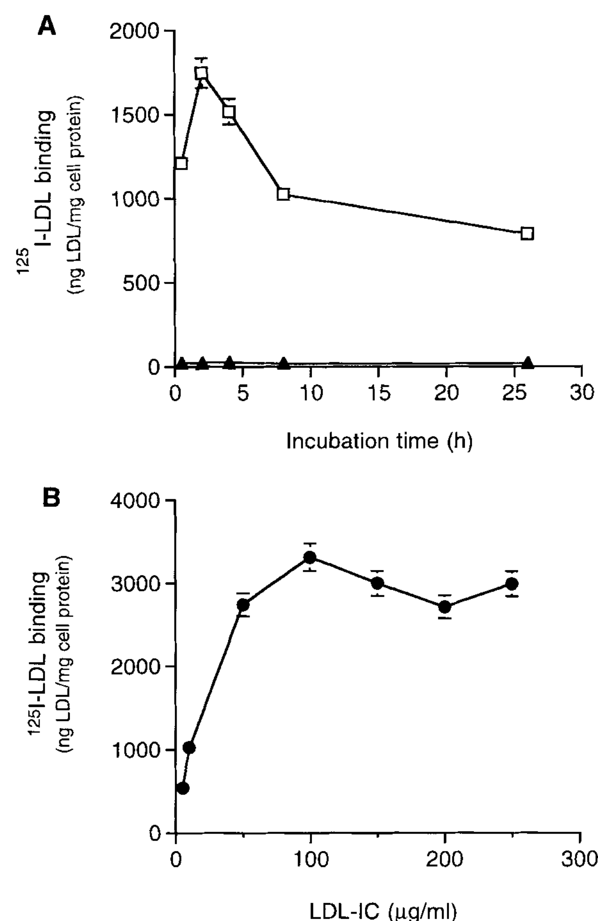


**Fig. 3.** Time course and concentration studies of receptor-mediated binding of $^{125}$I-labeled LDL by PMA-treated THP-1 cells after LDL-IC stimulation. A: PMA-treated THP-1 cells were incubated at 37°C with serum-free medium alone (▲) or SFM containing LDL-IC (150 μg/ml) (□) for the periods of time indicated in the figure. B: PMA-treated THP-1 cells were incubated for 2 h, at 37°C, with serum-free medium (SFM) or SFM containing LDL-IC at the concentrations indicated in the figure. After the incubation, binding was determined as described in the legend for Fig. 1. The results represent the mean ± SEM of three experiments, run in triplicate.

observed in PMA-treated THP-1 cells stimulated by LDL-IC was due to changes in the rate of LDL-R mRNA gene transcription, a nuclear run-on assay was performed. Nuclei were isolated from the LDL-IC-stimulated or unstimulated cells and in vitro transcription was carried out in the presence of $^{32}$P-UTP, as described in Material and Methods. The transcribed products were then analyzed by hybridization to denatured LDL-R, HMG-CoA reductase, and GAPDH cDNAs immobilized on nylon membranes. The results showed a 3-fold increase in the hybridization signal of LDL-R using transcription products from nuclei of LDL-IC-stimulated cells, as compared with those from nuclei of unstimulated cells (**Fig. 6**). In contrast and, as expected, the hybridization signal of HMG-CoA reductase was not significantly increased (Fig. 6). As the above data clearly



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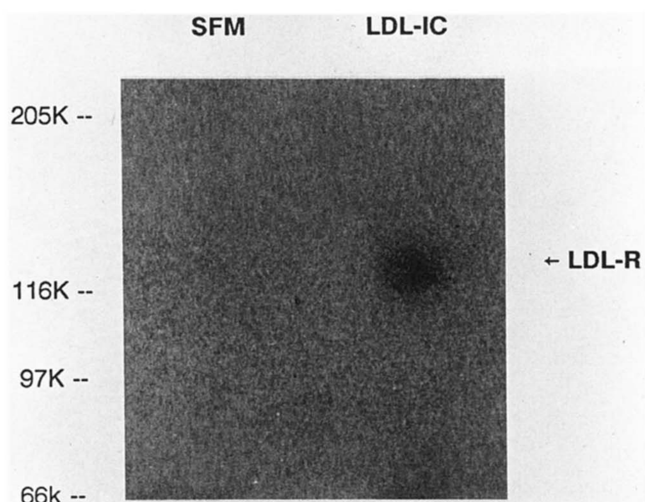


**Fig. 4.** LDL-R protein in LDL-IC-stimulated and unstimulated THP-1 cells determined by immunoblotting. PMA-treated THP-1 cells were incubated with SFM or SFM containing 150 μg/ml of LDL-IC for 2 h at 37°C. After the incubation the cells were washed three times with PBS, pH 7.4, and then lysed with a buffer containing Triton X-100 and protease inhibitors (see Methods). One hundred μg of protein was electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose. Immunoblotting was performed with an antibody recognizing the carboxyl-terminal peptide of LDL-R as described in Methods. The immunoreactive bands were identified by incubation with $^{125}$I-labeled anti-rabbit IgG and visualized by exposure to Kodak film for 18 h at −70°C. The relative electrophoretic mobility of molecular weight markers is indicated on the left.

showed that the increase in LDL-R protein (40-fold) was greater than that expected for the increase observed in LDL-R mRNA (7-fold) we determined the effect of LDL-IC on LDL-R mRNA stability. Our data showed that the degradation rates of LDL-R mRNA in both control and LDL-IC-treated cells were similar (**Fig. 7**), suggesting that LDL-IC stimulation did not alter LDL-R mRNA stability. The concentration of actinomycin D used in the above experiments was 5 μg/ml as this concentration was shown to inhibit 98% of [$^3$H] uridine incorporation into RNA.

As a change in LDL-R mRNA stability could not explain the marked increase in LDL-R protein, we determined next whether or not LDL-IC stimulation led to translational activation of LDL-R mRNA. Our data show that there was no increase in LDL-R protein when the cells were incubated with LDL-IC in presence of 10 μg/ml of cycloheximide (**Fig. 8**), suggesting that translational activation of LDL-R mRNA occurred during the stimulation of the cells with LDL-IC.

As shown in the initial experiments, the stimulation of PMA-treated THP-1 cells with LDL-IC led to a marked accumulation of CE in these cells and to their transformation into foam cells (Fig. 2 and Table 1). A substantial increase in free cholesterol was also observed in the same cells (Table 1). Regardless of the increase in free cholesterol content, these cells showed a significant increase in LDL-R transcription. To determine whether the increase in LDL-R transcription was secondary to a depletion in the cell cholesterol regulatory pool despite the increase observed in the cell total free cholesterol content, several experiments were performed. We started by repeating the LDL binding experiments in cells stimulated with LDL-IC in the presence or absence of 12 mM mevalonolactone and two different concentrations of 25 hydroxycholesterol (5 and 25 μg/ml of medium). The results of these experiments are summarized in **Fig. 9** and show that the addition of mevalonolactone, a precursor for the endogenous synthesis of cholesterol, and of 25-hydroxycholesterol to the LDL-IC-stimulated cells did not prevent the marked increase in LDL binding. In contrast, when mevalonolactone and 25-hydroxycholesterol were added to unstimulated cells, a marked drop of LDL binding (to 38% of baseline level) was observed. We then performed experiments where inhibitors of ACAT were used to prevent cholesterol esterification. Two different inhibitors were used: progesterone and the Sandoz compound 58-035. Binding of LDL to THP-1 cells stimulated with LDL-IC was markedly enhanced regardless of the addition of either of these two ACAT inhibitors to the medium **(Fig. 10).** Both of these compounds, however, in the concentrations used in our experiments, were able to markedly inhibit the incorporation of [$^{14}$C]oleate into cholesteryl esters in THP-1 cells incubated with LDL-IC. In the absence of the Sandoz compound 58-035 or progesterone, the THP-1 cells stimulated by LDL-IC incorporated 4531 pmol/of [$^{14}$C]oleate/mg cell protein per 18 h. When 1 μg of the Sandoz compound 58-035 was added to the medium simultaneously with LDL-IC, the incorporation of [$^{14}$C]oleate was reduced to 122.6 pmol/mg cell protein (97.3% inhibition). Similarly, when 300 μg of progesterone was added, the incorporation of [$^{14}$C]oleate dropped to 486.5 pmol/mg cell protein (89.3% inhibition). The concentrations of the ACAT inhibitors used in our experiments were previously determined to lead to maximal inhibition of [$^{14}$C]oleate incorporation into cholesteryl esters without inducing cell toxicity.

## DISCUSSION

It has been shown that human monocytic THP-1 cells, when exposed to phorbol ester treatment, stop dividing and quickly acquire macrophage-differentiated functions, including a marked decrease in LDL-R expression (14). In the present study, we have demonstrated that PMA-treated THP-1 cells, after stimulation with LDL-IC, ox-LDL-IC, and mda-LDL-IC, have the same alterations in lipid/lipoprotein metabolism that we have observed in human monocyte-derived macrophages, i.e., en-



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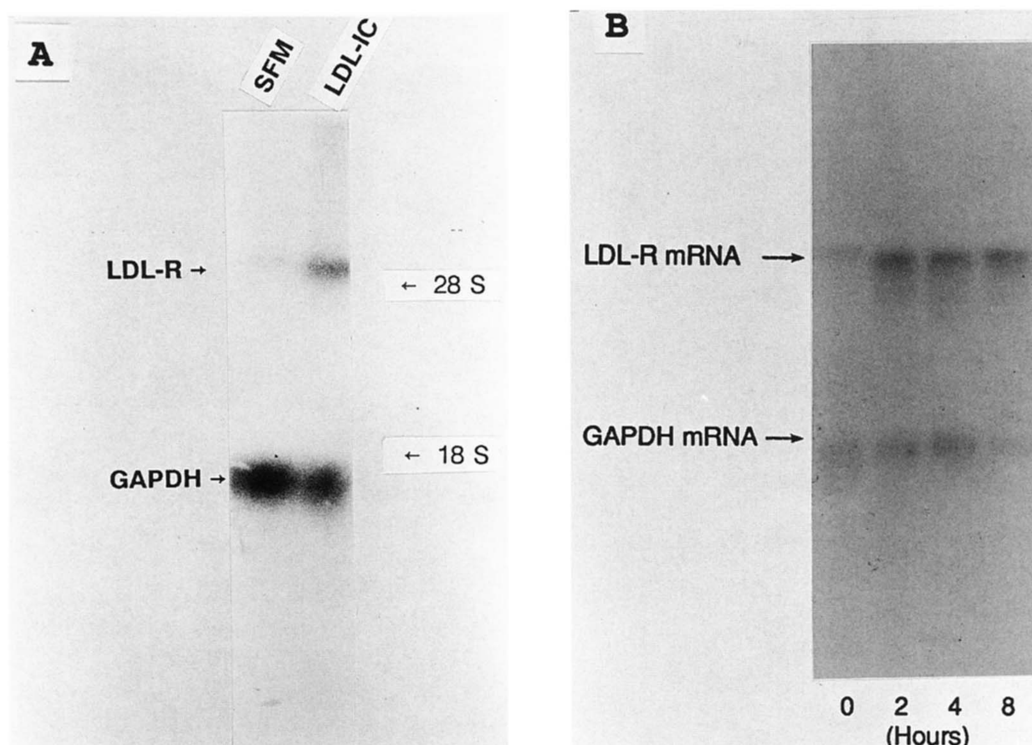


**Fig. 5.** Northern blot analysis of LDL-R mRNA in PMA-treated THP-1 cells stimulated with LDL-IC. A: PMA-treated THP-1 cells were incubated at 37°C with serum-free medium (SFM) or SFM containing 150 μg/ml of LDL-IC for 2 h. Total RNA isolated from the LDL-IC-stimulated and unstimulated cells was subjected to electrophoresis on 1% agarose/formaldehyde and transferred to a nylon membrane. The LDL-R and GAPDH mRNAs immobilized on the nylon membrane were hybridized with $^{32}$P-labeled LDL-R and GAPDH cDNAs as described in Methods. The relative concentration of LDL-R mRNA was estimated by densitometry of the autoradiograms and compared to that of GAPDH mRNA. B: PMA-treated THP-1 cells were incubated with 150 μg/ml of LDL-IC for different time periods as indicated. After incubation, total RNA was isolated and Northern blot was performed as described above and in Methods. This figure is representative of three different experiments.

hanced receptor-mediated binding of LDL and increased CE accumulation leading to their transformation into foam cells. By Western blot, using an antibody against the carboxyl-terminal domain of the LDL-R, we have also clearly demonstrated that the marked increase in receptor-mediated LDL binding was paralleled by a similar increase in LDL-R protein, thus confirming that the increase in LDL binding was secondary to an increase in LDL-R number as previously described (13).

TABLE 2. Densitometric scanning of the autoradiogram from Northern blot analysis

| | Relative Intensity | | |
|---|---|---|---|
| | SFM | LDL-IC | LDL-IC/SFM |
| LDL-R/GAPDH | 0.14 | 0.95 | 6.79 |
| HMG-CoA reductase/GAPDH | 0.24 | 0.25 | 1.04 |

PMA-treated THP-1 cells were incubated in the serum-free medium (SFM) with or without 150 μg/ml of LDL-IC at 37°C for 2 h. Total RNA was isolated and LDL-R, HMG-CoA reductase and GAPDH mRNA were analyzed by Northern blot as described in Methods. Densitometric scanning of the autoradiograms was performed to determine the relative intensity of the bands. An arbitrary unit of 1.0 was assigned to the background of the film. The data shown represent the average of two experiments.

As THP-1 cells were shown by the above experiments to be a good model for human monocyte-derived macrophages and both cholesterol loading and LDL-R upregulation were induced to a similar extent by immune complexes containing either native or oxidized LDL, all the experiments designed to investigate the molecular mechanisms responsible for the induced LDL-R expression were performed in PMA-treated THP-1 cells stimulated by LDL-IC.

There were two major advantages in using THP-1 cells to perform these experiments. THP-1 cells are easy to grow and thus it was technically easier and more economical to perform the experiments with these cells instead of human macrophages. Furthermore, due to the large amount of peripheral monocytes required for each experiment, the performance of the different experiments using cells from the same donor would have been impossible. In the other hand using different monocyte donors would not allow us to compare the findings of the



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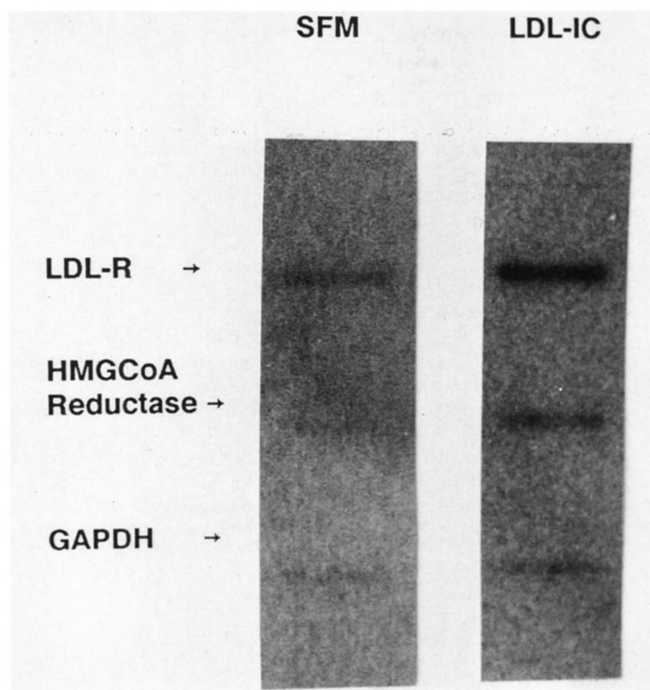


**Fig. 6.** Nuclear run-on analysis of transcriptional activity of LDL-R and HMG-CoA reductase in PMA-treated THP-1 cells stimulated with LDL-IC. PMA-treated THP-1 cells ($20 \times 10^6$) were incubated with serum-free medium (SFM) or SFM containing 150 μg/ml of LDL-IC (LDL-IC) for 6 h. Nuclei were prepared as described in Methods from stimulated or unstimulated cells for in vitro RNA synthesis. $^{32}$P-labeled RNA was then purified and hybridized to nylon membranes in which LDL-R, HMG-CoA reductase, and GAPDH cDNAs have been previously immobilized. The blot was autoradiographed and the film was subjected to densitometric scanning. The relative concentrations of LDL-R and HMG-CoA reductase mRNA from LDL-IC-stimulated and unstimulated cells were measured and compared to that of GAPDH mRNA. This is a representative experiment from a total of two experiments.

different experiments due to the variability in the degree of response between individual donors.

By Northern blot and nuclear run-on assays we have clearly demonstrated that there is an increase in the transcriptional activation of the LDL-R gene. LDL-R mRNA levels were increased from 2- to 7-fold after stimulation of the cells with LDL-IC for 2 h and that increase was maintained for several hours. Similarly, the nuclear run-on assay showed a 3-fold increase in the incorporation of $^{32}$P-UTP into the LDL-R mRNA. The above results demonstrated that increased transcription of the LDL-R gene is partially responsible for the increase in LDL-R protein but cannot, on its own, explain it. The binding and Western blot studies showed, on average, a 40-fold increase in receptor-mediated LDL binding (binding studies) and LDL-R protein (Western blot) in LDL-IC-stimulated cells as compared to unstimulated cells. The increase in LDL-R protein is, therefore, much higher than that of LDL-R mRNA. Thus, the increase in LDL-R expression induced by LDL-IC in PMA-treated

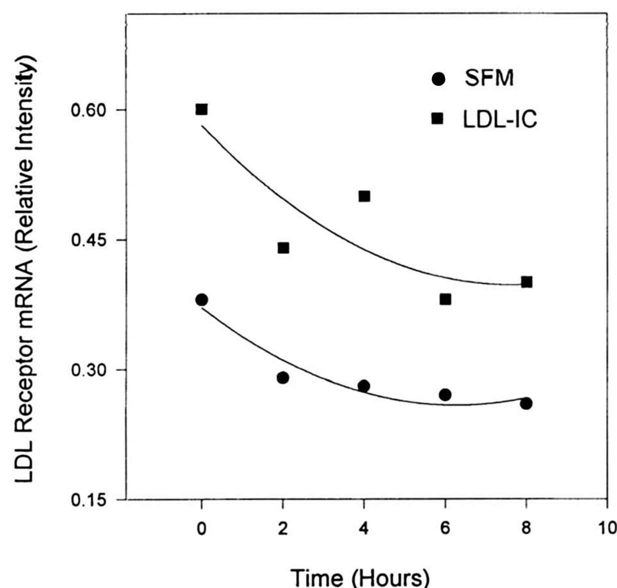


**Fig. 7.** Degradation of LDL-R mRNA in PMA-treated THP-1 cells. PMA-treated THP-1 cells were incubated, at 37°C, with serum-free medium (SFM) or SFM containing 150 μg/ml of LDL-IC for 2 h prior to the addition of 5 μg/ml of actinomycin D. The incubation was continued for 8 h afterwards. Total RNA was isolated before (0 time) and 2, 4, 6, and 8 h after the addition of actinomycin D. Slot-blot analysis of LDL-R mRNA was performed as described in Methods.

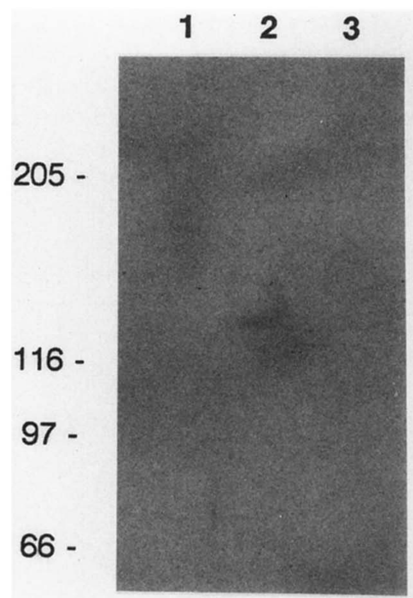


**Fig. 8.** The effect of cycloheximide on the up-regulation of LDL-R expression induced by LDL-IC. PMA-treated THP-1 cells were incubated with serum-free medium (SFM) (lane 1), or with SFM containing 150 μg/ml of LDL-IC in the absence of 10 μg/ml of cycloheximide (lane 2) or in the presence of cycloheximide (lane 3) at 37°C for 2 h. After the incubation, the cells were lysed and the lysates were subjected to Western blot analysis as described in Methods. Protein molecular weight markers are shown on the left.



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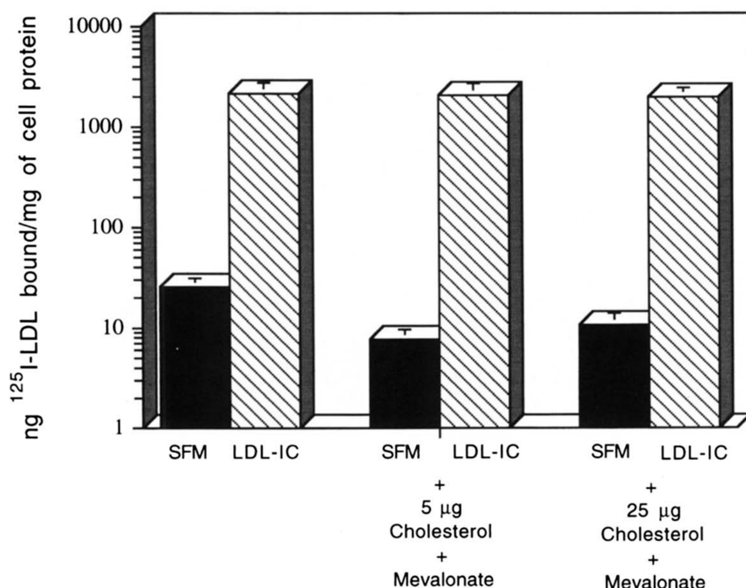


**Fig. 9.** Receptor-mediated binding of $^{125}$I-labeled LDL to THP-1 cells stimulated with LDL-IC in the presence or absence of mevalonic acid lactone and 25-hydroxycholesterol. PMA-treated THP-1 cells were incubated with SFM or with SFM to which 12 mM mevalonic acid lactone and either 5 or 25 µg/ml 25-hydroxycholesterol were added. To half of the wells containing the different additives, 150 µg/ml of LDL-IC was added and the cells were incubated for 2 h at 37°C. After this incubation the medium was removed, the cells were washed, and SFM was added to all the wells. The cells were then cooled to 4°C and 10 µg/ml of $^{125}$I-labeled LDL was added to all wells in the presence or absence of 250 µg/ml of unlabeled LDL to determine receptor-mediated binding (see Methods). The cells were incubated again at 4°C for 4 h and harvested afterwards for gamma counting and protein determination. The results represent the mean±SEM of three experiments run in duplicate.

THP-1 cells is secondary not only to the activation of transcription of the LDL-R gene but also to the activation of a post-transcriptional event. Possible post-transcriptional events include altered mRNA stability, translational activation, decreased protein degradation, and increased translocation to the membrane of LDL-R protein. As our Western blot in the unstimulated whole cell extract showed only trace amounts of LDL-R protein, it was unlikely that decreased degradation or increased translocation to the membrane of the LDL-R protein was responsible for the marked increase in LDL-R expression. Altered message stability and/or translational activation were the most likely post-transcriptional events to explain the increase in LDL-R protein. LDL-R mRNA stability was estimated by a inhibitor chase study that clearly showed that there was no change in the LDL-R mRNA degradation rate after LDL-IC stimulation. In contrast, the addition of cycloheximide completely abolished LDL-R expression in the LDL-IC-stimulated cells, suggesting that translation activation as demonstrated by increased de novo synthesis of LDL-R protein is likely the post-transcriptional event induced by LDL-IC stimulation.

Transcriptional and post-transcriptional regulation of LDL-R gene expression has been described previously. Vitols et al. (22) reported a multilevel regulation of the LDL-R gene expression in leukemic cells during cholesterol deprivation. They found that LDL-R activity in these cells increased 5-fold as compared to that in normal cells whereas LDL-R mRNA levels only in-

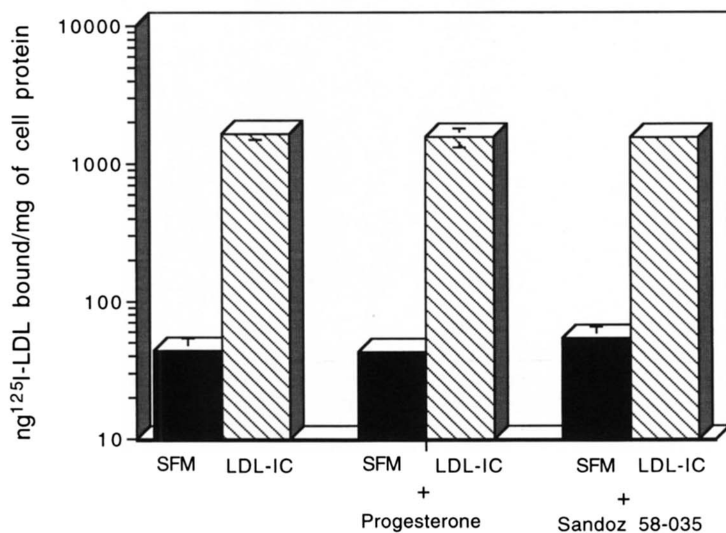


**Fig. 10.** Receptor-mediated binding of $^{125}$I-labeled LDL to THP-1 cells stimulated with LDL-IC in the presence or absence of two ACAT inhibitors: Sandoz compound 58-035 and progesterone. PMA-treated THP-1 cells were incubated with SFM or with SFM to which either 1 µg/ml of the Sandoz compound 58-035 or 300 µg of progesterone was added. To half of the wells containing the different additives, 150 µg/ml of LDL-IC was added and the cells were incubated for 2 h at 37°C. After this incubation the medium was removed, the cells were washed, and SFM was added to all the wells. The cells were then cooled to 4°C and 10 µg/ml of $^{125}$I-labeled LDL was added to all wells. To half of the wells in each treatment, 250 µg/ml of unlabeled LDL was added to determine nonspecific LDL binding. The cells were then incubated again at 4°C for 4 h and harvested afterwards for gamma counting and protein determination, as described in Methods. Receptor-mediated binding was determined as described in Methods. These results represent the mean±SEM of three experiments run in duplicate.



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creased 2-fold. The regulation of LDL-R gene expression that is not mediated by the transcriptional activation via the sterol regulatory element (SRE) has also been described by Sharkey et al. (23). They observed that the LDL-R activity was still sterol-responsive in receptor-deficient cells transfected with cDNA for the LDL-R but lacking the sterol regulatory element, suggesting that more mechanisms, including other regulatory element(s) in the LDL-R gene or translational activation, may be involved in the regulation of LDL-R gene expression. Multilevel regulation of gene expression other than LDL-R has also been described in several studies. For example, Mueckler, Merrill, and Pitol (24) described translational and pretranslational control of ornithine aminotransferase synthesis in rat liver. Guyette, Matusik, and Rosen (25) also demonstrated a prolactin-mediated transcriptional and post-transcriptional regulation in the expression of the casein gene. Therefore, multilevel control of gene expression is not an uncommon regulatory event and it is present in the biosynthesis of many proteins.

As regulation of LDL-R transcription has been closely related to the presence of an intracellular regulatory pool of free cholesterol, we decided also to investigate whether the increased transcriptional activity of the LDL-R gene was secondary to a decrease in the cell sterol regulatory pool. Although THP-1 cells after stimulation with LDL-IC are transformed into foam cells and contain a significant increase in free cholesterol, the possibility that during cell activation most of the free cholesterol is used for membrane synthesis or is deviated from the regulatory pool to the ACAT pool for CE synthesis cannot be excluded. If that is the case, deprivation of free cholesterol in the regulatory pool may occur and lead to an increase in the transcriptional activity of the LDL-R gene.

We performed experiments to explore these two possibilities. If the reason behind depletion of free cholesterol in the regulatory pool was increased need for membrane synthesis, adding mevalonate and 25-hydroxycholesterol to the medium should obviate the problem. On the other hand, if deviation of free cholesterol to the ACAT pool was the major reason for the cholesterol depletion in the regulatory pool, adding ACAT inhibitors to the culture medium should resolve the problem. Progesterone and the Sandoz compound 58-035 were chosen for the experiments. The first compound has been shown to re-direct free cholesterol from the ACAT pool to the LDL-R regulatory pool; in contrast, the Sandoz compound 58-035 will not re-direct free cholesterol to the LDL-R regulatory pool. Our data showed that no inhibition in the receptor-mediated binding of LDL was observed in the cells stimulated with LDL-IC when 25-hydroxycholesterol and mevalonic acid lactone were added to the medium. In contrast, and as expected, in the unstimulated cells a decrease in receptor-mediated LDL binding was clearly seen when the above compounds were added to the culture medium. Similarly, no inhibition in the receptor-mediated binding of LDL was observed either in LDL-IC-stimulated or unstimulated cells when either of the two ACAT inhibitors mentioned above was added to the culture medium.

These data, as a whole, strongly suggest that in THP-1 cells stimulated with LDL-IC, the cell free cholesterol content does not play a role in the up-regulation of LDL-R expression. It also clearly shows that the increase in transcription of the LDL-R is not secondary to a decrease in the intracellular sterol regulatory pool.

Wang et al. (26) have recently shown that the cell sterol regulatory pool inhibits the activity of CPP32, a cysteine protease protein that is responsible for the cleavage of the sterol regulatory element binding protein (SREBP) 1 and 2. The cleavage of these proteins leads to the formation of a protein with lower molecular weight that binds to the sterol regulatory element (SRE), a 10-base pair element in the 5′ flanking region of LDL-R gene, and enhances transcription of the LDL-R gene. Our studies examining the effect of cell cholesterol content on the LDL-R expression do not exclude the possibility that activation of the cell by LDL-IC may lead to a marked increase in the activity of either SREBP or CPP32 that totally overrides the inhibition of CPP32 induced by the sterol regulatory pool. Alternatively, it is possible that the mechanisms involved in the increased transcription of the LDL-R gene are totally independent of the binding of these proteins to the SRE.

The fact that LDL-IC did not induce HMG-CoA reductase gene expression does not exclude the possibility that an increase in activity of either SREBP or CPP32 is behind the up-regulation of LDL-R expression, as recently, Wang et al. (27) have shown that the region that is necessary for sterol-mediated regulation in the HMG-CoA reductase gene loosely resembles the SRE but cannot be bound by SREBP.

In conclusion, we have proved that PMA-treated THP-1 cells are a good model for studying the up-regulation of LDL-R expression induced by LDL-IC. We have also demonstrated that the up-regulation of LDL-R expression in these cells is due not only to activation of the transcription of the LDL-R gene but also to a post-transcriptional event, most likely translation activation. Furthermore, we excluded the free cholesterol regulatory pool content as a possible reason behind the up-regulation of LDL-R expression in the same cells. Mechanisms responsible for the increased transcriptional activation of the LDL-R gene during cell activation by LDL-IC are presently under investigation in our laboratory.



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