Synergistic upregulation of low-density lipoprotein receptor activity by tamoxifen and lovastatin

Yajaira Suárez a,1, Carlos Fernández a,1, Diego Gómez-Coronado a, Antonio J. Ferruelo a, Alberto Dávalos a, Javier Martínez-Botasa, Miguel A. Lasunción a,b,*

a Servicio de Bioquímica-Investigación, Hospital Ramón y Cajal, Ctra. de Colmenar, km 9, E-28034 Madrid, Spain
b Departamento de Bioquímica y Biología Molecular, Universidad de Alcalá, 28771 Alcalá de Henares, Spain

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Abstract

Objective: To study the mechanism involved in the cholesterol-lowering activity of tamoxifen, an estrogen receptor (ER) modulator widely used in breast cancer therapy.

Methods and results: We used MOLT-4 cells, which do not express estrogen receptors and require important amounts of cholesterol for proliferation. We firstly confirmed that tamoxifen reduced cholesterol biosynthesis by inhibiting sterol D8,7-isomerase and D24-reductase activities, which resulted in the accumulation of zymosterol. In cells incubated in the presence of low-density lipoprotein (LDL) (120 µg cholesterol/ml), tamoxifen stimulated LDL receptor activity and expression in a dose-dependent manner, as determined by 1,1'-dioctadecyl-3,3,3,3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled LDL uptake, LDL receptor expression on the cell surface and LDL receptor mRNA levels. Furthermore, tamoxifen, but not lovastatin, inhibited the egress of LDL-derived cholesterol from lysosomes, as ascertained by filipin staining in both MOLT-4 and HepG2 cells. When studied in combination, especially at relatively high LDL concentrations in the medium, tamoxifen and lovastatin stimulated LDL receptor activity synergistically, which is attributed to the different mechanism of action these drugs exhibit.

Conclusions: The present study demonstrates the stimulation of the LDL receptor by tamoxifen. These results explain the long-known hypolipidemic effect of tamoxifen and support its use, or that of other intracellular cholesterol trafficking inhibitors, in combination with statins for the reduction of plasma LDL cholesterol levels.

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1. Introduction

Tamoxifen is an estrogen receptor (ER) modulator widely used in breast cancer therapy, which displays tissue-specific estrogen agonist/antagonist properties [1]. In addition, treatment with tamoxifen was observed to reduce both cholesterol levels [2,3] and mortality from cardiovascular causes [4], and there is ample evidence on its protection from atherosclerosis [5–7]. The underlying mechanisms, however, appear to be diverse. Tamoxifen has been shown to inhibit cholesterol biosynthesis [8–10], to inhibit acyl-CoA: cholesterol acyltransferase [11], to interact with P-glycoprotein [12], a protein involved in intracellular cholesterol trafficking [13], and to protect low-density lipoprotein (LDL) from peroxidation [14,15], a mechanism that also could account for its cardioprotective action. As regards to LDL receptor activity, previous studies by others have reported conflicting results, either a stimulation [16], an inhibition [10,17] or no effect [18]. Very recently, Brüning et al. showed that tamoxifen stimulates LDL receptor gene expression by activating the
binding of the ER-α/Sp1 complex to the sterol regulatory cis-element (SRE) present in the promoter [19].

In the present work, we studied the effects of tamoxifen on LDL receptor in human MOLT-4 cells, which do not express ER [13], and the results were compared to those of lovastatin, which stimulates LDL receptor expression as a result of hydroxymethylglutaryl (HMG)-CoA reductase inhibition. We found that tamoxifen prevents the down-regulation of the LDL receptor induced by LDL cholesterol. In combination with lovastatin, the stimulation of LDL receptor activity was synergistic. In addition to the inhibition of cholesterol biosynthesis, tamoxifen interfered with the egress of LDL-derived cholesterol from the lysosomal compartment to the endoplasmic reticulum, where the sterol sensing machinery resides [20]. This explains the persistent stimulation of LDL receptor gene transcription despite important amounts of LDL cholesterol were being taken up.

2. Materials and methods

2.1. Materials

MOLT-4 (human lymphoblastoid) (ATCC CLR 1582) and HepG2 (human hepatocarcinoma) (ATCC HB 8065) cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Fetal bovine serum (FBS), normal goat serum (NGS), RPMI 1640, DMEM, Trizol reagent and antibiotics were obtained from Gibco-BRL (Barcelona, Spain). Lipoprotein-deficient serum (LPDS) was prepared from FBS by ultracentrifugation at a density of 1.21 kg/l. Anti-LDL receptor monoclonal antibody (IgG C7), Ready-To-Go DNA labeling beads kit, M-labeled anti-mouse IgG and labelled IgG C7, Ready-To-Go DNA labeling beads kit, M-labeled anti-mouse IgG and labelled IgG C7 were gifts from Zeneca Pharma (Wellesley, MA, USA). Filipin and poly-l-lysine were from Boehringer Mannheim Biochemica (Barcelona, Spain). 1,1-Dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI) and 3,3-dihexiloxocarbocyanine iodide (DiOC6) were from Molecular Probes Europe (Leiden, Netherlands). Dual Luciferase Reporter Assay System, pRL-CMV, random hexamers, MMLV RT and RNAsin were from Promega (Madison, WI, USA). Taq polymerase and dNTPs were from Perkin Elmer (Wellesley, MA, USA). Filipin and poly-l-lysine were provided by Sigma (St. Louis, MI, USA). Tamoxifen, lovastatin and GF120918 were gifts from Zeneca Pharma (Madrid, Spain) and Glaxo-Wellcome (Madrid, Spain), respectively. The other chemical products were of analytical grade.

2.2. Cell culture

MOLT-4 and HepG2 cells were maintained in RPMI 1640 and DMEM, respectively, supplemented with 10% FBS and antibiotics, at 37 °C in a humidified atmosphere of 5% CO2.

For experiments, cells were incubated in their respective medium containing 10% LPDS and supplemented with known amounts of human LDL cholesterol.

2.3. [14C]-Acetate incorporation into sterols

MOLT-4 cells (1.5×10⁵/ml) were incubated in RPMI 1640, 10% LPDS containing 20 μCi/ml of [14C]-acetate for 8 h, at 37 °C in 5% CO2, and the cellular sterols were analyzed by HPLC, as described [21].

2.4. LDL receptor activity assays

Human LDLS were isolated and labelled with the fluorescent probe DiI as reported [22]. MOLT-4 cells (0.4×10⁵ cells/ml) in RPMI 1640 containing 10% FBS or LPDS supplemented or not with LDL, were treated with vehicle, tamoxifen and/or lovastatin for 24 h. Then, cell viability was assessed by Trypan blue exclusion and, subsequently, the cells were washed and resuspended (1×10⁵ cells/ml) in fresh medium containing Dil-LDL (30 μg cholesterol/ml). Non-specific uptake was determined in extra tubes containing a 50-fold excess of unlabelled LDL. The cells were incubated for 2 h at 37 °C to allow Dil-LDL uptake. In other instances, cells were incubated for 24 h with different Dil-LDL concentrations simultaneously with the drugs under study. In all cases, the cells were washed and resuspended in 1 ml of phosphate-buffered saline (PBS) containing 20 nM DiOC6 and then incubated at 37 °C for 10 min. This fluorochrome is a Δψm-sensitive dye that allows to distinguish between live and apoptotic cells [23]. After washing, cells were analyzed by flow cytometry (FACScalibur, Becton Dickinson). The results are expressed in terms of specific median intensity of fluorescence (M.I.F.) in arithmetic/linear scale in living cells (DiOC6 positive) after subtracting autofluorescence of cells incubated in the absence of Dil-LDL.

2.5. Expression of the LDL receptor on the cell surface

After incubation with the drugs, the cells were washed and resuspended (5×10⁵ cells/ml) in PBS containing 1% NGS. Then, the cells were incubated for 15 min at room temperature, anti-LDL receptor monoclonal antibody IgG C7 (15 μg/ml) was added and incubation was prolonged for 1 h on ice. Subsequently, the samples were washed with PBS and incubated in ice-cold PBS, 1% NGS and FITC-labeled goat anti-mouse IgG for 30 min. Finally, the samples were washed, resuspended in 1 ml of PBS and analyzed by flow cytometry. The results are expressed in terms of M.I.F. after subtracting the autofluorescence of the cells.

2.6. Northern blot

Total cellular RNA was extracted with Trizol reagent. RNA (15 μg) was denaturated with formaldehyde and
subjected to 1% agarose gel electrophoresis in the presence of formaldehyde. The RNA was transferred to nylon membranes by capillary blotting with 20×SSC for 18 h and fixed by UV irradiation. The LDL receptor cDNA probe (800 bp) was prepared by digesting the plasmid pLDLR2 (kindly provided by Dr. Pocovi, Universidad de Zaragoza, Spain) with endonuclease PstI, and labeled with [32P]-dCTP by using Ready-To-Go DNA labeling beads kit. The labeled probe was separated from unincorporated nucleotides by Microspin S-300 HR columns. Prehybridization was performed in prehybridization–hybridization buffer (50% deionized formamide, 6×SSC, 5× Denhardt, 0.5% SDS, 100 µg/ml herring sperm) for 2 h at 42 °C. The blots were hybridized overnight at 42 °C, washed twice in 2×SSC with 0.1% SDS at room temperature for 15 min, followed by two washes in 0.1×SSC, 0.1% SDS at 55 °C, and two 15-min high stringency washes with 0.1×SSC, 0.1% SDS at 65 °C. Finally, the filters were exposed to Kodak X-omat-film at −70 °C. Ethidium bromide stained 28S ribosomal RNA bands were used to normalize the LDL receptor mRNA expression [24].

2.7. RT-PCR analysis

cDNA was synthesized from RNA samples by mixing, in a total volume of 25 µl, 2 µg of total RNA and 500 ng of random hexamers in the presence of 200 U MMLV RT, 30 µM RNAsin, 15 mM dNTPs and 50 mM Tris–HCl buffer (pH 8.3). Samples were incubated at 37 °C for 60 min. A 2-µl aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers. Each 50 µl PCR contained 2 µl of the RT reaction, 1 mM dNTPs, 20 pmol of each primer, 1.25 U of Taq DNA polymerase and 20 mM Tris–HCl, pH 8.5. The sequences of the sense and antisense primers used were: LDL receptor (NM_000527), 5′-ACTGCGAAGATGGCTGGATG-3′ and 5′-CATCTGAC-CAGTCCGGGCAGT-3′; hydroxymethylglutaryl (HMG)-CoA reductase (NM_00859), 5′-CTGACAA-GAAAACCTGCTGCTGCT-3′ and 5′-CGGTCCACACAATCCGG-3′; ABCA1 (NM_005502), 5′-GGAGGCAATGCGACTGAGGAA-3′ and 5′-CCTGCGTTTGCGTGAAGTGT-3′; GAPDH (BC_013310), 5′-CCACCCATGGCGACATTCCATGGC-3′ and 5′-TCTAGAGCGGTCGATGCCCACC-3′. PCR was performed for 29 (LDL receptor), 26 (HMG-CoA reductase), 32 (ABCA1) or 22 (GAPDH) cycles. Each cycle consisted of denaturation at 90 °C for 1 min, primer annealing at 60 °C for 1 min, and primer extension at 72 °C for 2 min. PCR reaction products were separated on a 2% agarose gel, and stained with ethidium bromide.

Fig. 1. Effects of tamoxifen and lovastatin on [14C]-acetate incorporation into sterols. (A) MOLT-4 cells were treated with 5 µM tamoxifen, 1 µM lovastatin or vehicle (control) and [14C]-acetate for 8 h, and radioactivity incorporation into sterols was determined by HPLC. (1) Cholesterol; (2) zymosterol. (B) Scheme of the cholesterol biosynthesis pathway showing the enzymes inhibited by tamoxifen.
2.8. Plasmid constructs, transient transfections and measurement of luciferase activity

The plasmid construct TATA-pXP2 was prepared replacing the polylinker region of the promoterless luciferase reporter vector pXP2 [25] with a similar polylinker containing at its 3' end a TATA box correctly located with respect to the transcription start site. The plasmid construct SRE-TATA-pXP2 was generated by cloning a double stranded oligonucleotide containing two tandem copies of the SRE region (5'-AATCACCCCACTGCAAACTC-CTCCCCCTGC-3') described for the promoter of the LDL receptor gene [26], into the KpnI and BglII sites of the TATA-pXP2 vector. MOLT-4 (4×10⁶ cells) cultured in RPMI 1640, with 10% FBS were transfected with the plasmid SRE-TATA-pXP2 and with vector pRL-CMV as described [27]. Aliquots of the transfected cells were subjected to the different drug treatments and then assayed for luciferase and Renilla activities [27].

2.9. Filipin staining and fluorescence microscopy

Cells were cultured on glass coverslips, previously treated with poly-d-lysine, and stained with filipin for free cholesterol as described [28].

2.10. Statistical analysis

The results are expressed as mean±S.D. Statistical comparisons between groups were done by the Student’s t test or analysis of variance (ANOVA) and post hoc multiple comparisons with the Student–Newman–Keuls test, by using the Statgraphics Plus v5.0 program (Statistical Graphics, USA).

3. Results

3.1. Effect of tamoxifen on cholesterol biosynthesis

MOLT-4 cells were incubated in a cholesterol-deficient medium containing [¹⁴C]-acetate and simultaneously treated with the drugs. As shown in Fig. 1A, 5 μM tamoxifen suppressed radioactivity incorporation into cholesterol, but increased its appearance in the precursor zymosterol. This is consistent with the inhibition of both Δ²4-reductase and Δ²27-isomerase by tamoxifen (Fig. 1B). Just for comparison, 1 μM lovastatin totally abolished [¹⁴C]-acetate conversion into any sterol (Fig. 1A), which is in accordance with the inhibitory action on HMG-CoA reductase.

3.2. Effects of tamoxifen on LDL receptor activity

To study the effects of tamoxifen on LDL receptor activity, different approaches were undertaken. Firstly, we analyzed the specific uptake of Dil-LDL in cells incubated in a 10% FBS-containing medium (final total-cholesterol concentration, 45 μg/ml). As shown in Fig. 2A, tamoxifen clearly stimulated LDL receptor activity in a dose-dependent manner, up to a concentration of 5 μM (178±23.8% of the control). As expected, lovastatin also stimulated this activity, being equally effective at 1, 2 and 5 μM (approximately 130%) (Fig. 2A). To ascertain whether these changes in Dil-LDL uptake correlated with the expression of the LDL receptor on the cell surface, the cells were immunostained with an anti-LDL receptor antibody and analyzed by flow cytometry. As shown in Fig. 2B, LDL receptor expression levels paralleled Dil-LDL uptake in the different assayed conditions (Fig. 2A).

Cholesterol concentration and distribution among lipoproteins vary among FBS batches. Since cholesterol availability is the major regulator of LDL receptor activity, we next examined the effects of the drugs in cells...
incubated in a more defined medium, such as 10% LPDS supplemented with a known concentration of LDL cholesterol: 120 μg/ml, which is one tenth of the normal LDL cholesterol concentration in human plasma. As shown in Fig. 3A, in the absence of any drug in the medium (vehicle), supplementing with LDL cholesterol for 24 h resulted in an intense repression of LDL receptor activity (28.9±13.8% of that in cells incubated in the absence of LDL-control). This down-regulation of LDL receptor was partially prevented by treatment with 5 μM tamoxifen (63.4±14.2% of the control) (Fig. 3A), whereas lovastatin had no appreciable effect (Fig. 3A). Expression of the LDL receptor on the cell surface paralleled the changes in DiI-LDL uptake (Fig. 3B). These results demonstrate that tamoxifen prevents the suppression of LDL receptor induced by LDL cholesterol in MOLT-4 cells. Similar results were obtained with HepG2 cells (data not shown).

When tamoxifen and lovastatin were added to the culture medium in combination, DiI-LDL uptake was three times higher than that in the absence of drugs, reaching a value similar to that found in cells incubated in the absence of LDL (Fig. 3A). Similar synergistic effects were observed for LDL receptor protein expression on the cell surface (Fig. 3B). Therefore, treatment with tamoxifen and lovastatin in combination totally abolished the down-regulation of LDL receptor induced by LDL cholesterol.

To further analyze the changes in LDL receptor activity, cells were incubated for 24 h in the presence of increasing concentrations of DiI-LDL and cell-associated DiI-fluorescence was determined at the end of the incubation period (Fig. 4). In control conditions, DiI-LDL uptake showed a saturation kinetics, reaching a plateau with approximately 30 μg/ml DiI-LDL cholesterol, which is in accordance with the well-known kinetic characteristics of the LDL receptor [29]. Lovastatin slightly increased DiI-LDL uptake, especially when lipoproteins were added at low, subsaturating concentrations, while tamoxifen intensely enhanced this uptake at any LDL concentration. Most interestingly, when combined with tamoxifen, lovastatin produced additive effects at each LDL concentration. Indeed, with 120 μg/ml DiI-LDL cholesterol, the uptake attained in cells treated with tamoxifen plus lovastatin was almost three times higher than that reached in the control or in cells treated with lovastatin alone (Fig. 4). These results unambiguously demonstrate the strong stimulatory effect of tamoxifen on LDL uptake.

Fig. 3. Effects of tamoxifen and lovastatin on LDL receptor activity and LDL receptor protein expression on the cell surface in cells incubated in LPDS. MOLT-4 cells were incubated in RPMI 1640 containing 10% LPDS (control, C) or supplemented with 120 μg/ml LDL cholesterol, and treated with vehicle (–), tamoxifen (TMX) or lovastatin (LOV) for 24 h, as indicated, and then used for LDL receptor activity as measured by DiI-LDL uptake (A) or LDL receptor expression on the cell surface with anti-LDL receptor antibody and flow cytometry analysis (B). Data are expressed as percentage of the control (10% LPDS in the absence of any drug). Mean±S.D. of five independent experiments. Statistical comparisons of the different treatments versus vehicle by Student’s t test: **p<0.01, ***p<0.001.
Fig. 5. Effects of tamoxifen and lovastatin on gene expression. MOLT-4 cells were incubated in RPMI 1640 containing 10% LPDS (control, C) or supplemented with 120 μg/ml LDL cholesterol, and treated with vehicle (–), tamoxifen or lovastatin for 24 h, as indicated. (A) Representative Northern blot and quantification of LDL receptor mRNA levels after normalizing by 28S ribosomal RNA (means of two independent experiments). (B) Representative RT-PCR for LDL receptor, HMG-CoA reductase, ABCA1 and GADPH, and quantification after normalizing by GADPH as reference (means±S.D. of three experiments).

Statistical comparisons between groups by ANOVA and Student–Newman–Keuls analysis; different letters denote statistically significant differences (p<0.05).
3.3. Effects of tamoxifen on gene expression

We next examined LDL receptor expression by measuring LDL receptor mRNA levels. As shown in Fig. 5A, LDL cholesterol reduced LDL receptor mRNA levels, as measured by Northern blot, and both tamoxifen and lovastatin partially prevented this effect. Similar results were obtained by RT-PCR (Fig. 5B). Treatment with the two drugs in combination abolished the inhibition of LDL receptor gene expression induced by LDL cholesterol (Fig. 5B).

We also analyzed the effects of tamoxifen, as compared to lovastatin, on the expression of other genes involved in cholesterol homeostasis, such as the HMG-CoA reductase gene, which encodes an enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis, and the ABCA1 gene, encoding a transporter implicated in cholesterol efflux. The former gene is transcriptionally regulated by cholesterol in a similar manner to the LDL receptor gene [30]. Consistently, supplementing the medium with LDL cholesterol resulted in the expected repression of HMG-CoA reductase gene expression, and the effects of the drugs alone or in combination paralleled those on LDL receptor (Fig. 5B). Transcription of ABCA1 has been shown to increase in response to cholesterol loading [31], which is apparently mediated by oxysterols activating LXR [32]. In line with this, supplementing the medium with LDL cholesterol increased ABCA1 mRNA levels (Fig. 5B). Interestingly, tamoxifen, but not lovastatin, prevented this effect.

SRE-binding proteins (SREBP) play a key role in the regulation of LDL receptor gene expression [20]. To analyze whether the observed effects on LDL receptor mRNA levels corresponded to changes in SREBP-mediated transcriptional activity, we transiently transfected MOLT-4 cells with a SRE-luciferase reporter gene and the effects of the drugs on its transcription were determined. As shown in Fig. 6, LDL cholesterol repressed SRE-driven transcription, whereas both tamoxifen and lovastatin partially prevented this effect. Likewise, when both drugs were used in combination, synergistic effects on the promoter activity were observed (Fig. 6). These results suggest that the effects of tamoxifen, both alone or in combination with lovastatin, on LDL receptor activity and expression are mediated by SREBP-driven transcription.

3.4. Tamoxifen causes lysosomal storage of LDL cholesterol

SREBP is activated in response to a reduction of free-cholesterol content in the endoplasmic reticulum [20]. To investigate whether the opposing effect of tamoxifen on the inhibition of LDL receptor by LDL cholesterol was due to an interference with intracellular free-cholesterol transport, the cells were stained with filipin and analyzed by fluorescence microscopy. This approach has been used by others to examine the accumulation of free cholesterol in late endosomes/lysosomes [33]. As shown in Fig. 7A, in cells incubated with LDL, treatment with tamoxifen caused an increase in bright perinuclear granules and decreased plasma membrane staining, indicative of free cholesterol accumulation in late endosomes/lysosomes. These effects were even more apparent in cells incubated with tamoxifen plus lovastatin (Fig. 7A). Interestingly, in cells incubated with Dil-LDL and treated with tamoxifen, filipin staining co-localized with DiI, as shown in both MOLT-4 and HepG2 cells (Fig. 7B), indicating that accumulated cholesterol derives from internalized LDL. These results demonstrate that tamoxifen interferes with the egress of free cholesterol from endosomes/lysosomes.

4. Discussion

In this study, we demonstrate that tamoxifen stimulates the expression and activity of the LDL receptor. These effects appear to be due to the inhibition of both cholesterol biosynthesis and intracellular cholesterol trafficking. The study was firstly undertaken with MOLT-4 cells, which do not express estrogen receptors [34] and require important amounts of cholesterol for membrane formation [35], and the results were confirmed with HepG2 cells. At a concentration of 5 μM—which is considered therapeutically relevant [36]—we found that tamoxifen practically blocked cholesterol biosynthesis and resulted in the accumulation of zymosterol, which agrees with the inhibitory of Δ8,7-isomerase and Δ24-reductase as reported by others [8–10].

In medium containing a low concentration of lipoprotein cholesterol (10% FBS), both tamoxifen and lovastatin significantly increased Dil-LDL uptake and cell surface
LDL receptor expression, which is consistent with the inhibition of cholesterol biosynthesis. Supplementing the medium with LDL produced the expected down-regulation of the LDL receptor. Interestingly, tamoxifen suppressed these effects of LDL, increasing both LDL receptor gene transcription, cell-surface expression and activity. Lovastatin did stimulate LDL receptor gene transcription, as measured by both Northern blot and RT-PCR, but seemingly did not increase LDL receptor activity. It is important to note that the former assays give cumulative measurements of gene transcription during the whole 24-h study period, whereas the LDL receptor activity assay and cell-surface protein expression, which are performed once completed the 24-h incubation, give a point measurement of the processes at that time. A similar disparity between LDL receptor mRNA and protein levels after treatment with statins has been also reported by other authors [37,38].

Regarding tamoxifen, the most striking finding was that while it highly enhanced LDL uptake, this was not followed by a substantial reduction of LDL receptor expression. In other words, it appears that tamoxifen inhibits the suppression of the LDL receptor induced by LDL. In order to regulate LDL receptor expression, LDL-derived cholesterol must reach the endoplasmic reticulum, where the SREBP/SCAP tandem resides [20]. We thus investigated the LDL receptor pathway and found that tamoxifen induced the accumulation of LDL-derived cholesterol in late endosomes/lysosomes. The retention of free cholesterol in this compartment is consistent with the observed stimulation of the expression of both LDL receptor and HMG-CoA reductase and the

Fig. 7. Lysosomal accumulation of cholesterol in cells treated with tamoxifen and/or lovastatin. (A) MOLT-4 cells were incubated in RPMI 1640, 10% LPDS, supplemented with 120 μg/ml LDL cholesterol, and treated with the drugs for 24 h. Cells were fixed and stained with filipin and photographed using a fluorescence microscope. (B) MOLT-4 and HepG2 cells were incubated simultaneously with DiI-LDL and 5 μM tamoxifen, and both filipin and DiI fluorescences were analyzed.
inhibition of ABCA1 by the effect of tamoxifen. Progesterone, which inhibits intracellular cholesterol trafficking [39], has been described to block the ability of LDL cholesterol to down-regulate HMG-CoA reductase in cultured cells [40]. Similar effects on LDL receptor activity were observed by others in both cells treated with U18666A (3β-dihydroxy-5-en-17-one hydrochloride) [28,41], and Nieman-Pick C (NPC) 1 or NPC2 negative cells [42], where the egress of LDL-derived cholesterol from lysosomes is handicapped. Therefore, the stimulatory action of tamoxifen on LDL receptor expression appears to reside on the reduction of cholesterol availability for the endoplasmic reticulum, as a result of the inhibition of both cholesterol biosynthesis and arrival of LDL-derived cholesterol from lysosomes. This mechanism may operate simultaneously with the direct stimulation of LDL receptor gene expression through an ER-α/Sp1 complex as shown in ER-expressing cells [19], both contributing to explain the hypolipidemic effect of tamoxifen.

It was reported by others that tamoxifen inhibits P-glycoprotein [12,43], a protein that has been involved in cholesterol trafficking from the plasma membrane to the endoplasmic reticulum [13]. In our system, however, P-glycoprotein seems not to mediate the action of tamoxifen herein reported, since the specific P-glycoprotein inhibitor, GF120918 [44], used at a wide concentration range (0.1–10 μM), did not stimulate LDL receptor activity (data not shown). This is consistent with the fact that non-stimulated MOLT-4 cells barely express P-glycoprotein [45]. Other authors also excluded a role for P-glycoprotein in the intracellular transport of endocytosis-derived cholesterol regardless the expression of MDR1 [46].

The combination of tamoxifen and lovastatin resulted in a synergistic stimulation of the LDL receptor activity, which is consistent with their distinct mechanisms of action. Since tamoxifen permitted the synthesis of zymosterol, the additional increase of LDL receptor expression induced by lovastatin probably resides in the block of sterol synthesis this drug produces. Based on these results, the simultaneous inhibition of sterol biosynthesis and intracellular cholesterol trafficking appears to be an efficient way to maximally stimulate LDL receptor activity.

In spite of its hypolipidemic effect, use of tamoxifen for the treatment of hypercholesterolemia is a matter of controversy, owing to the side effects that this drug produces [47]. Clinical studies should evaluate the potential benefit of the combined administration of cholesterol trafficking inhibitors and statins for the treatment of severe hypercholesterolemia.

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References


[24] Dahl DM, Gillespie DD, Sulser F. Ethidium bromide fluorescence of 28S ribosomal RNA can be used to normalize samples in northern or dot blots when analyzing small drug-induced changes in specific mRNA. J Neurosci Methods 1992;42:211–8.


