ELSEVIER

Contraception 84 (2011) 160 – 168

Contraception

Original research article

Effect of a combination oral contraceptive (desogestrel+ethinyl estradiol) on the expression of low-density lipoprotein receptor and its transcription factor (SREBP2) in placental trophoblast cells☆

Albina Arjuman, Hemlata Pandey, Nimai Chand Chandra*

Department of Biochemistry, All India Institute of Medical Sciences, Ansari Nagar, New Delhi-110 029, India

Received 6 October 2010; revised 23 November 2010; accepted 30 November 2010

Abstract

Background: This in vitro study deals with the effect of a combination oral contraceptive steroid — desogestrel and ethinyl estradiol — on the expression of low-density lipoprotein receptor (LDLR) and its transcription factor (SREBP2) in assessing the functional effectiveness of the LDLR.

Study design: Differentiated primary placental trophoblast cells isolated from term human placentae and cells from Jar cell line were used for the study. Low-density lipoprotein receptor and SREBP2 expressions were assessed by immunocytochemistry and immunoblot assays with and without combination contraceptive steroid challenge. Functional activity of LDLR was studied by rating the profile of cellular uptake of fluorescent Dil-LDL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanin perchlorate-LDL). Quantitation of Dil-LDL was done spectrofluorometrically.

Results: Variation of concentration(s) of either of the components of a combination preparation (desogestrel and ethinyl estradiol) showed a comparable change in the expressions of LDLR and SREBP2 to attain their optimal levels. Maximum expression and a significant functional effectiveness were observed at a unique combination of desogestrel (20 ng/mL) and ethinyl estradiol (10 ng/mL).

Conclusion: The stimulatory effect of a combination contraceptive steroid on LDLR expression is an associated phenomenon of the contraceptive-mediated stimulation of SREBP2 expression.

Keywords: Oral contraceptive; Desogestrel; Ethinyl estradiol; LDL receptor; SREBP2

1. Introduction

Estrogen(s) and progestogen(s) are known to have antagonistic effects on lipid metabolism [1–3]. Their combination is demonstrated here in the formulation of an oral contraceptive (OC). The third-generation OCs in combination with less androgenic derivative of progestogen, viz., desogestrel, have shown a prodigious impact as well as therapeutic controversy regarding lowering the possibilities of coronary heart disease [4,5]. One of the major causes of heart attack is hyperlipoproteinemia, which results from inadequate clearance of low-density lipoprotein (LDL) from blood vessels mainly by LDL receptors (LDLRs) [6–8] in association with other scavenger receptor(s) [9–11]. It is well known that LDLR is one of the most widely distributed cellular endocytic receptor [12–14] for lowering LDL concentration in blood vessels. A previous report from our laboratory [15] had shown that a combination of desogestrel (20 ng/mL medium) and ethinyl estradiol (EE, 10 ng/mL medium) maintained the LDLR at much higher than normal levels of expression and functioning mode in placental cells. It has been shown by Brown and Goldstein [16] and Shea-Eaton et al. [17] that the expression of LDLR is maintained by the SREBP2 (sterol regulatory element binding protein), a transcription factor for LDLR gene-mediated feedback

☆ This study was funded by the Indian Council of Medical Research, Government of India. A.A. and H.P. were supported by Research Assistant Fellowships from the Indian Council of Medical Research, India.

* Corresponding author. Tel.: +91 11 26594267; fax: +91 11 26588641, 26588663.

E-mail address: chandra_nc1@rediffmail.com (N.C. Chandra).

0010-7824/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.contraception.2010.11.020

regulation. It is not known whether the effect of combination OC on LDLR is also guided by the same SREBP2 modulated signaling mechanism or is a completely independent phenomenon.

Among the extrahepatic tissues, LDLR is found to be abundant in steroidogenic organs and in the placenta. In the placenta, more localization of LDLR is found in trophoblast cells. Since direct evidence regarding human tissue cells can be obtained by evaluating LDLR expression in human placental cells, trophoblast cells from human placenta (placenta is obtained as a rejected tissue after delivery) have been considered for the present study. A placental trophoblast cell line, JAR (ATCC HTB-144), is also used for comparison. Different concentrations of components in the combination OC (desogestrel+EE) have been utilized in the present study to see their effects on the expression of LDLR and its transcription regulatory protein SREBP2.

2. Materials and methods

2.1. Chemicals

Cerazette (0.075 mg desogestrel tablet) and Lynoral (0.01 mg EE tablet) were obtained from Organon (India) Limited, Kolkata, India. DMEM/F-12 Nutrient Mixture was obtained from GibcoBRL, New York, NY, USA. Fetal calf serum (FCS), antibiotic antimycotic solution (100×), acrylamide/bis-acrylamide and HEPES were purchased from Sigma Chemical Co., St. Louis, MO, USA. Low-density lipoprotein receptor goat polyclonal primary antibody (N-17, sc-11822, epitope at the N-terminus of LDLR of human origin) was obtained from Biotechnology Inc., Santa Cruz, CA, USA. Anti-goat HRP-conjugated secondary antibody and nitrocellulose membrane were purchased from Genotech, St. Louis, MO, USA. FemtoLUCENT detection kit was purchased from Biotechnology Inc., Santa Cruz, CA, USA. DAB (peroxidase substrate kit) and R.T.U. Vectastain universal quick kits (streptavidin/peroxidase complex and biotinylated pan-specific antibody) were procured from Vector Laboratories, Inc., Burlingame, CA, USA. Plastic wares for cell culture were from Becton Dickinson Labware, Bedford, MA, USA. All other chemicals used were of analytical reagent grade.

2.2. Cell line culture

JAR cells were obtained from the repository of the National Centre for Cell Science, NCCS Complex, University of Pune Campus, Ganesh Khind, Pune, India. Cells were grown in RPMI 1640 medium with L-glutamine (GibcoBRL) containing $NaHCO_3$ (2 g/L) and 10% FCS (vol/vol). Penicillin (100 U/mL), streptomycin (100 mcg/mL) and amphotericin B (250 ng/mL) were included in the cell cultures. The cells were initially grown at 37°C in an atmosphere of 95% air and 5% CO_2 on 25-cm^2 flasks and then seeded in 90-mm dishes in the same environment of temperature and gassing for the following studies.

2.3. Primary culture of trophoblast cells from human placenta

Discarded human term placentas were obtained from uncomplicated vaginal delivery or from cesarean section at the Department of Obstetrics and Gynaecology, All India Institute of Medical Sciences, New Delhi, after institutional ethics approval. The placentae were transported to the laboratory in sterile normal saline within 30 min of delivery. Approximately 30–40 g of saline-washed villous tissue was processed aseptically for isolation of trophoblast cells. In brief, the tissue was minced, transferred to a digestion flask containing 150 mL of warm calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS) with 26 mM HEPES, 0.125% trypsin 1:250 and 0.2 mg/mL DNase I (150K U/mg), pH 7.4, and incubated in an orbital shaking water bath at 37° for 30 min. The top liquid was decanted, and the remaining placental tissue was subjected to the same digestion procedure. This process was repeated thrice and the pooled digest (liquid part from three digestions) was centrifuged at 1000×*g* for 10 min at room temperature in 50-mL polystyrene centrifuge tube(s) by layering 45 mL of the digest over 5 mL of FCS in each tube. The resultant pellet(s) (isolated cells) was resuspended in 5 mL DMEM/F-12 nutrient medium and kept at room temperature (Sol-A). A Percoll solution (Sol-B) was prepared by mixing nine parts of Percoll Stock (Sigma) with one part of 10× HBSS. The Sol-B was used to develop a continuous gradient (70% to 5%) in 1× CMF-HBSS in 50-mL conical polystyrene centrifuge tube. The cell suspension (Sol-A) was subjected to density gradient centrifugation at 1200×*g* in room temperature for 20 min by carefully layering upon the Percoll density gradient column. Following centrifugation, the middle layer of the gradient was found to contain a relatively uniform population of mononuclear cells. The fraction corresponding to the density of 1.048–1.062 containing approximately 98% pure population of cytotrophoblast cells [18] was carefully aspirated out and washed once again with DMEM/F-12 nutrient medium.

Percoll purified trophoblast cells were diluted to a concentration of 10^6 cells/mL with DMEM/F-12 nutrient medium containing 10% FCS and antibiotic concentration as mentioned above. The cells were then plated on 1% gelatin/poly-L-lysine-coated cover slips placed in wells of a 12-well plate and maintained at 37°C in a humidified atmosphere. One set of Percoll purified cells was maintained for not more than 6 h in culture, which served as undifferentiated control cells, i.e., native cytotrophoblast. The other set of cytotrophoblast cells in culture was maintained for 36 h until they differentiated towards an extravillous anchoring phenotype, i.e., mature syncytiotrophoblast cells. Following this, both sets of plates were

washed with fresh medium to remove the nonadherent cells and processed for immunocytochemical studies.

2.4. Contraceptives

Desogestrel and EE pills were crushed and then dissolved separately in minimal quantity of 50% ethanol (vol/vol). The concentration of desogestrel in the stock preparation was determined by measuring absorption at 250 nm and comparing the result against a standard curve made from absorption maxima of various concentrations of medroxyprogesterone at 250 nm. Concentration of EE (absorption maxima at 280 nm) was also obtained in the same way by comparing with the standard curve of β-estradiol at 280 nm. Working stock dilution to the respective concentrations was prepared in medium which was then used directly in the culture medium to acquire the required contraceptive concentration. Control cells received only a medium having equivalent concentration of ethanol-vehicle.

Based on previously published work from our lab [15], the concentration limit of the component contraceptives used in the medium was 20 ng/mL of desogestrel and 10 ng/mL of EE, which correlates with the adult human dose of 0.1 mg desogestrel and 0.05 mg EE in OC pills. This dose was determined considering an average total 5 L blood volume in an adult human.

A dose of 0.1 to 0.15 mg desogestrel has been used as progestogen component in certain OC pills. An amount of 0.05 mg of EE has been used in some of their OC preparation. The mean relative bioavailabilities, in blood, of desogestrel (measured as 3-keto-desogestrel, the active metabolite of desogestrel) and EE from the combination tablet were found to be 100% and 93%, respectively [19].

2.5. Steroid challenge

2.5.1. Trophoblast cells

The cells were cultured on a 12-mm cover glass coated with 1 mg/mL solution of poly-L-lysine. The lysine-coated cover glasses were placed under medium in the wells of a 12-well plate. The initial number of cells added was 5×10^5 cells/mL medium. These cells were allowed to grow for 36 h and then incubated with fresh serum-free F-12 medium with and without contraceptives. The specific doses of contraceptives in the medium were decided on the basis of the prevalent human dose used in 5 L blood volume. An equal volume of 50% ethanol in the medium was used corresponding to each dose of contraceptive as control. The cells were incubated at 37°C for 2 h on a rocker platform, following which they were washed with fresh medium and subjected to the following experimental analysis.

2.5.2. Placental JAR cell

JAR cells were grown to about 70% confluence in 90-mm Petri plates, following which the contraceptive challenge was given in serum-free medium and cells incubated for 2 h at 37°C on a rocker platform. Following this, the cells were washed, collected and lysed. The lysate was used for subsequent Western blot analysis.

2.6. Preparation of JAR cell lysate

The cells from each plate were lysed by strong vortexing in 100 μL of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 0.5% (vol/vol) Triton X-100, 5 mM EDTA with 2 mM PMSF, 10 mcg/mL leupeptin and 10 U/mL aprotinin. The lysed suspension was kept on ice for 30 min and then spun at 10,000×*g* for 15 min at 4°C. The supernatant was collected, and protein content was determined.

2.7. Immunocytochemistry

Trophoblast cells were cultured on 12-mm microscopic cover glass slips flooded with medium in a 12-well plate, following which they were incubated with different doses of contraceptives in DMEM/F-12 medium for 2 h at 37°C on a rocker platform. The cells were then washed with 10 mM phosphate-buffered saline (PBS) and fixed in chilled acetone at 4°C for 10 min. Following this, they were treated with 4% H_2O_2 in methanol for 30 min at room temperature; this inhibited endogenous peroxidase activity. The cells were rinsed in 10 mM PBS containing 0.01% Triton-X (PBST) for 5 min. Nonspecific binding was blocked using 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Three washes with PBST were repeated. This was followed by 2-h incubation with primary antibody against LDLR or SREBP-2 at a dilution of 1:25 in 10 mM PBS. The cells were washed thrice in PBST followed by incubation with specific biotinylated secondary antibody for 1 h at room temperature. The cells were then incubated for 1 h with avidin-HRP after a prewash in PBST and then treated with DAB substrate (0.06% DAB, 50 mM Tris–HCl, pH 7.6, 1% H_2O_2) for 2–3 min at room temperature. Finally, cover glasses were rinsed in distilled water and counterstained with Mayer's hematoxylin for about 1 min; excess stain was rinsed with water, air dried, dehydrated in xylene for 5 min and mounted on a DPX mountant. Photographs were taken after observation at 20× magnification by a CoolSNAP-Procf color digital camera attached to an Olympus U-CMAD3 microscope. An image analysis system was used to see the integrated optical density (IOD) of the stained cells. A minimum of 50–300 cells were analyzed by the image analyzer. The image analysis system consisted of a research microscope (BX50; Olympus, Tokyo, Japan), 10-bit digital camera (Xilix Correco, Canada), image grabber card (F-64, Cerreco Corp., Quebec, Canada) and a personal computer (P-III; Digital Corp., CA, USA). The image analysis software used was Optimas 5.2 (Optimas Corp., CA, USA).

2.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Electrophoresis was carried out on 10% polyacrylamide gels (1.5 mm thick), overlaid by a 4% stacking gel in sodium dodecyl sulfate according to the standard procedure of

Laemmli [20]. The proteins were transferred onto nitrocellulose membranes [21]. The membranes were incubated with blocking buffer [5% Blot-Quick blocking power (Genotech) in 10 mM PBS containing 0.05% Tween-20] for 2 h at room temperature on a shaker. The membrane was then washed thrice in 10 mM PBS–0.1% Tween-20 for 10 min each. Anti-LDLR polyclonal primary antibody [Santa Cruz Biotechnology, Inc., N-17, sc-11822] was added at a dilution of 1:1000 and kept for 2 h at room temperature on a shaker. The wash was repeated, and rabbit-antigoat-HRP-conjugated polyclonal secondary antibody (Bangalore Genei, India) was then added at 1:10,000 dilution and kept for 2 h at room temperature followed by three washes in PBS–Tween buffer. The blot was then developed by the femtoLUCENT detection kit (Santa Cruz, CA, USA). Protein expression was evaluated by determining the intensity of darkness of protein bands by a densitometer (Alpha Imager EC Gel Doc System, CA, USA) using Alpha Imager software.

2.9. Isolation of LDL

Low-density lipoprotein was isolated from human plasma by NaCl-KBr density gradient centrifugation according to Havel et al. [22]. Low-density lipoprotein was dialyzed in 10 mM PBS at 4°C before use. Human plasma used for isolating LDL was freshly collected from human blood obtained from the Blood Bank-Main Hospital of All India Institute of Medical Sciences maintaining the ethics of the institute body.

2.10. Fluorescent (Dil) labeling of LDL

Low-density lipoprotein was mixed with Dil [30 mg of Dil (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanin perchlorate) in DMSO stock solution] in a ratio of 1 mg Dil for 100 mg LDL protein in the dark. This mixture was incubated at 37°C for 18 h. Following incubation, its density was raised from 1.006 to 1.063 by addition of NaCl–KBr salt solution as shown by Havel et al. [22]. This sample was then loaded into the tubes and layered with the equivalent-density salt solution and centrifuged at 105,000×*g* for 22 h at 15°C. The labeled LDL fraction that appeared at the top of the tube was collected and dialyzed similarly as shown by Havel et al. [22]. The Dil-LDL was then quantified based on total protein estimated by Bradford's method [23]. After protein estimation, the Dil-LDL sample was diluted in saline to 100, 500 and 1000 ng/mL. One milliliter of isopropanol was added to each of them and mixed thoroughly. The isopropanol fraction was then used for estimating the quantity of incorporated Dil by measuring the absorbance in a spectrofluorometer with the excitation and emission wavelengths set at 520 and 574 nm, respectively. One hundred nanograms per milliliter Dil in isopropanol was used as standard and isopropanol as blank. All samples were taken in duplicates, and the readings were used to estimate the amount of Dil/mg of LDL protein [24].

2.11. Dil-LDL uptake study

To meet sufficient cell numbers, which are not possible to get from primary placental cell culture, the uptake study using Dil-LDL was carried out on JAR cells seeded in the wells of standard 12-well culture plates at cell density of 2×10^5 cells/well. The cells were incubated with varying concentrations of Dil-LDL (one concentration in one well and in triplicate) at 37°C for 5 h. After 5 h, the externally adhered LDL on the surface of the cells was removed by treating cells with dextran sulfate buffer (50 mM NaCl, 10 mM HEPES, 10 mg/mL dextran sulphate) for 1 h at 4°C. Then medium was removed. After washing the cells with physiological saline, isopropanol (95%) was added and incubated for 15 min at room temperature. The isopropanol was collected and centrifuged at 5000×*g* for 10 min at room temperature. The supernatant was used to quantify Dil fluorometrically as above.

2.12. Protein estimation

Protein estimation was done by the Bradford method [23] using BSA as the standard.

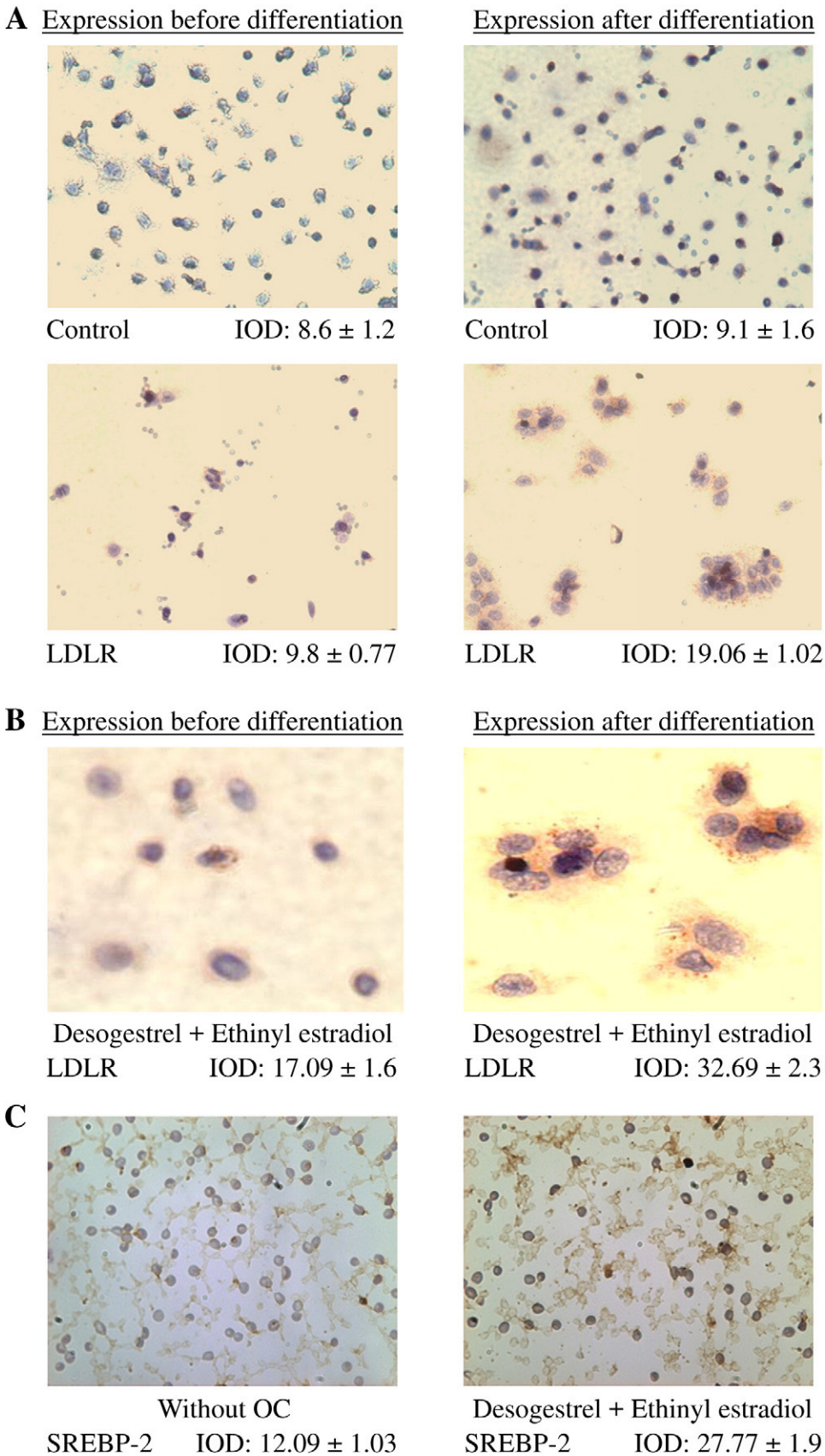
2.13. Study design

The whole study was done in two groups. In one group, primary placental trophoblast cell culture was used for study. This primary culture was developed from placentae obtained from different human donors. On the other hand, a stable placental trophoblast cell line was used to compare the data obtained from primary cultures. This comparison reflected the consistency of the result obtained by the use of combination OC.

3. Results

3.1. Expression of LDLR and SREBP2 by placental trophoblast cells in primary culture

Primary trophoblast cell culture was prepared by isolating cells from freshly obtained human placental tissue and culturing them in DMEM/F12 growth medium. These cells underwent differentiation in culture medium after 36 h from initial plating time. Therefore, the profile of expressions of LDLR and its transcription factor SREBP2 was checked in undifferentiated and differentiated placental trophoblast cells. The baseline expression was examined in the absence of any steroid component of the OC used for the study, and controls were made using the vehicle (50% ethanol) instead of any primary antibody. The quantitative measure of the expression profile was carried out by estimating the IOD (see Material and Methods) value of the DAB-conjugated brown-colored ligand–antibody complex obtained by immunocytogram. A minimum of 50 cells were analyzed by image analysis software from each experimental batch, and standard division was calculated from the results of 3 to 5 such experiments. Since it was not possible


A
Expression before differentiation
Expression after differentiation
Control
IOD: 8.6 ± 1.2
Control
IOD: 9.1 ± 1.6
LDLR
IOD: 9.8 ± 0.77
LDLR
IOD: 19.06 ± 1.02
B
Expression before differentiation
Expression after differentiation
Desogestrel + Ethinyl estradiol
LDLR
IOD: 17.09 ± 1.6
Desogestrel + Ethinyl estradiol
LDLR
IOD: 32.69 ± 2.3
C
Without OC
SREBP-2
IOD: 12.09 ± 1.03
Desogestrel + Ethinyl estradiol
SREBP-2
IOD: 27.77 ± 1.9

to get primary culture for all our experiments from cells isolated only from one placenta, cells were isolated time to time from placentae available from different subjects and each time fresh culture was prepared for experiments. Since cells from different placentae were not exactly identical, morphological changes exist in cells from one to another experiment. Our results showed that postdifferentiated cells expressed more LDLRs as compared to undifferentiated cells (Fig. 1A). Our previous report [15] showed a profoundly higher expression of LDLR by 20 ng/mL of desogestrel in combination with 10 ng of EE/mL medium. Using the same combination OC mix here, the present study also showed higher LDLR expression and comparatively more with postdifferentiated cells than undifferentiated ones (Fig. 1B).

Since feedback regulation of LDLR expression is maintained by the endoplasmic reticulum-mediated transcription factor SREBP2 [16], we compared the expression levels of LDLR and SREBP2 in differentiated primary placental trophoblast cells by evaluating the IOD value obtained for respective stained protein in immunocytogram. Fig. 1C shows that the increased expressions of SREBP2 in differentiated cells by the combination OC, used per mL medium, were comparable with those of LDLR found in differentiated cells in the presence of combination OC (Fig. 1B). This result supports the earlier report that expression of LDLR is a posttranslational phenomenon of SREBP2 [16,25,26], and it is a unique feature even when LDLR is stimulated by the steroid components of combination OCs.

3.2. Expression of LDLR and SREBP2 by cells from JAR cell line culture

As primary cultures were not developed from cells of a single placental origin and since each placenta was obtained from a different donor, multifactorial (heredity, age, stress, diet, etc.) variations could not be ruled out to influence gene expression. At least a change in cellular morphology was reflected in the above immunocytograms.

In order to avoid such anomalies, we verified the above results with a stable placental trophoblast cell line (JAR cell line) culture (Fig. 2A, B). Here we examined the effects of variation of component steroid concentrations in the combination OC on the expressions of LDLR and SREBP2 by immunoblot assay. Like before, here also we found a parallel change in the expression profile between LDLR and SREBP2. In Fig. 2A, desogestrel concentration was kept constant at 20 ng/mL, and EE concentration was varied from 0 to 10 ng/mL. In Fig. 2B, EE was kept constant at 10 ng/mL, and desogestrel was varied from 0 to 20 ng/mL. In both cases, the densitometry showed that the change of expression of LDLR maintained a similar trend with that of SREBP2 and attained the maximum peak at the ratio of 20 ng/mL desogestrel to 10 ng EE/mL medium. The consistency of results was determined from the significant statistical evaluation of data obtained by three repeated experiments. This again showed that the increased expression of transcription factor SREBP2 extends a parallel effect in the expression of LDLR on cell surface.

3.3. Dil-LDL uptake by placental cells

Since enough cells were not available in primary cultures to carry out LDL-uptake study at several LDL concentrations in the medium, the placental trophoblast cell line (JAR) was used for this purpose. Utilization of extracellular LDL by placental trophoblast cells from JAR cell line culture was observed in the presence and absence of the combination OC. Low-density lipoprotein was labeled with fluorescent dye Dil, a probe to detect the internalized LDL within the cell. Our results from three consecutive studies (Fig. 3) showed more clearance of extracellular LDL by LDLR with the combination OC (20 ng desogestrel/mL with 10 ng EE/mL medium) as compared to the case with no OC, and the enhanced activity of LDLR was also statistically significant. This explains the fact that highly expressed LDLRs in combination OC-treated cells also enhanced their functional ability to clear more extracellular LDL particles as compared to untreated cells.

4. Discussion

After almost 15 years of use in clinical practice, side effects of OCs were detected among the users in the years of 1975–1982 [27–31]. Besides many others, cardiovascular abnormalities having vascular thrombosis, viz., arterial and venous thromboembolism, along with myocardial infarction were the major ones. To eliminate such probabilities, several trials were undertaken in the following years by changing the component steroids and their doses (estrogen and progestogen components). These trials developed the second-generation OC with lesser steroid components and then the third generation by using less androgenic derivatives of progestogen in addition to low concentration of component steroids in the OC composition [32–34]. In our previous report [15], we showed that the combination of

Fig. 1. Immunocytochemistry. Expressions of LDLR and SREBP2 were compared in primary placental (human) trophoblast cell culture. (A) Expression of LDLR by primary placental trophoblast cell culture: shows higher expression of LDLR in differentiated cells. (B) Expression of LDL receptor by primary placental trophoblast cell culture incubated with combination OC: shows more stimulation of LDLR expression in differentiated cells by combination OC. (C) Expression of SREBP2 by differentiated primary placental trophoblast cell culture [incubated with and without OC]: an increased expression of SREBP2 by combination OC with differentiated cells. The expressions of LDLR and SREBP2 were evaluated from mean IOD±SD of cells after analyzing a minimum of 50 cells by the image analysis software of each category.

20 ng desogestrel with 10 ng EE had a potential of keeping LDLRs in the mode of high expression and functional effectiveness. Although it is known that saturation of cells with steroid decreases LDLR expression [16], estrogen as a mitogen may also enhance LDLR expression in its own limit [15]. After many trials in our previous study [15], we compromised the concentration of the progestogen and estradiol concentrations in 5 L volume (approximate adult blood volume) to 0.1 mg desogestrel (66% less of 0.15 mg; the upper limit used in third-generation OC) and 0.05 mg EE (66% more of 0.03 mg; the upper limit used in third-generation OC) to maintain LDLRs at both higher expression and functionally maximal state. The concentration of combination OC per 1 mL medium (20 ng desogestrel+10 ng EE) used in this study was equivalent to the above combination calculated for 5 L blood volume. Low-density lipoprotein receptors are the well-known cholesterol-clearing proteins present on the surface of all cells except RBC and are engaged in clearing extracellular cholesterol by the mechanism called endocytosis. Since the concentration of vessel cholesterol is one of the determining factors in developing vascular thrombosis, the extent of LDLR expression and its functional ability play a critical role in controlling cardiac disease and associated anomalies.

Although in our previous report [15] we claimed that the combination OC (desogestrel+EE) could keep LDLR in a highly expressed state and in super functional mode, we

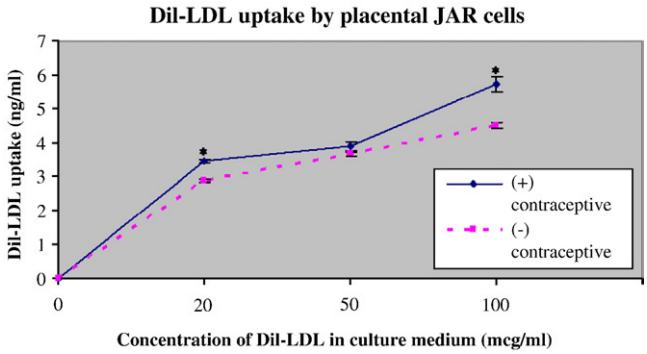


Fig. 3. Dil-LDL uptake assay. Assay was performed on JAR cells with and without combination OC. Dil-LDL uptake was estimated fluorometrically. *x*-axis: increasing concentration of Dil-LDL in incubation medium. *y*-axis: net uptake of Dil-LDL by cells from the medium. Amount (ng/mL) of uptake was calculated from the estimated fluorescence of the Dil internalized into the cells. All values are mean±SD (triplicate at each concentration). Statistical analysis by Student's unpaired *t* test. ∗p<.01.

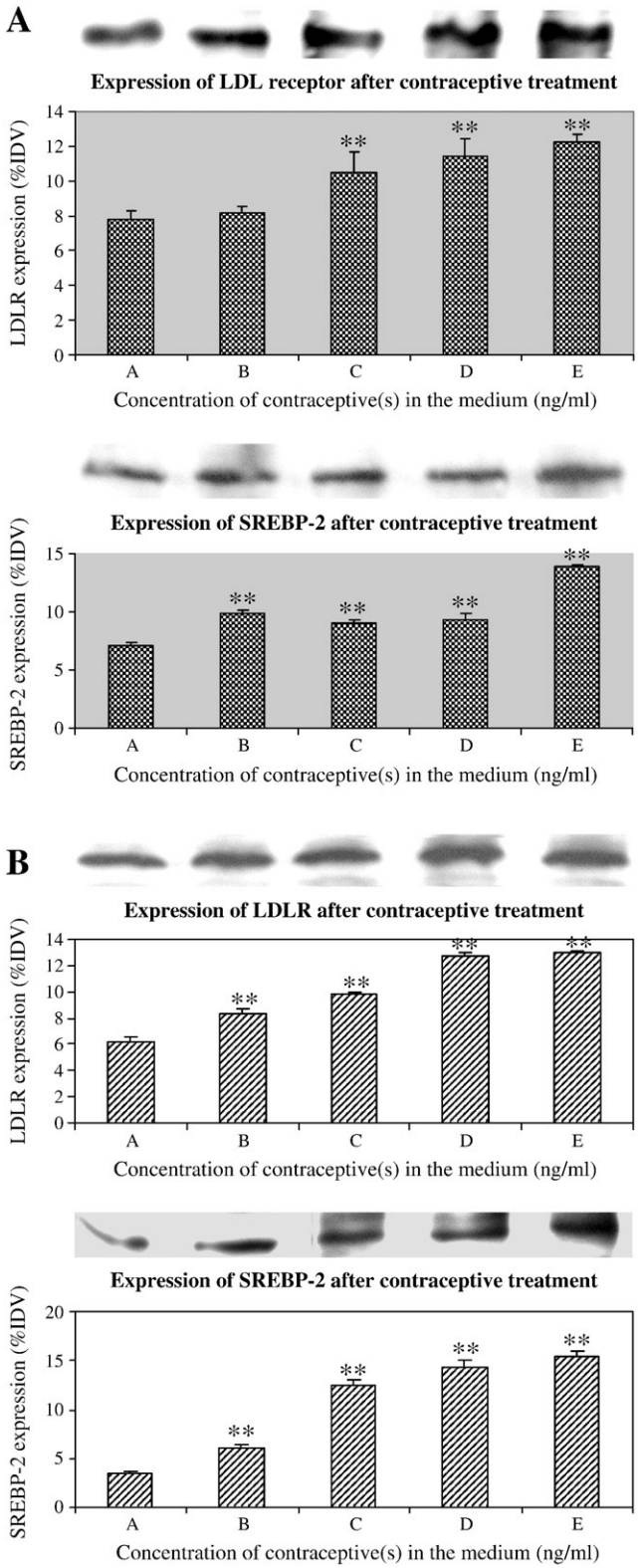


Fig. 2. Immunoblot. Expression of LDLR and SREBP2 in JAR cells with change of concentration of component contraceptives. Densitometry of the bands was performed and % IDV (integrated density value) was calculated. β-Actin was used as internal control (not shown). All values are mean±SD (triplicate at each concentration). Statistical analysis by one-way analysis of variance followed by Dunnett's multiple comparison. ∗∗p<.01. (A) Desogestrel is constant at 20 ng/mL, and EE is varying from 0–10 ng/mL. Ethinyl estradiol: A: 0 ng/mL, B: 1 ng/mL, C: 2.5 ng/mL, D: 5 ng/mL, E: 10 ng/mL. (B) Ethinyl estradiol is constant at 10 ng/mL, and desogestrel is varying from 0 to 20 ng/mL. Desogestrel: A: 0 ng/mL, B: 5 ng/mL, C: 10 ng/mL, D: 15 ng/mL, E: 20 ng/mL.

could not provide any reason behind it. Previous reports from Brown and Goldstein [6,8,16] and other laboratories [35,36] have confirmed that the transcription of LDLR is regulated by intracellular cholesterol saturation as well as by transcription factor SREBP2 [37,38]. Therefore, our aim in this study was to see whether there was any relation between OC-induced expression of LDLR and SREBP2. We examined this phenomenon by taking placental trophoblast cells from two different origins — the differentiated primary culture from human placenta and a stable cell line (JAR) culture.

We have explored different concentrations of components in the combination OC (desogestrel+EE) to see their effects on the expression of LDLR and its transcription regulatory protein SREBP2. The present study has reconfirmed our previous report [15] that a combination of 20 ng desogestrel and 10 ng EE per mL medium presents maximum LDLR expression on placental trophoblast cell. The stimulation of LDLR by OC has also been supported by the profile of SREBP2 expression. Similar effect may be expected with other tissue cells, but intracellular saturation by sterol components over a period of time might have an inhibitory effect on LDLR expression as was shown in our previous report [15,39] and elsewhere [8].

The differentiated trophoblast cells in the primary culture from human placenta showed comparatively more LDLR expression as compared to undifferentiated cells. Therefore, the differentiated cells were used to compare the effect of the combination OC on LDLR and SREBP2. It was found that the combination OC steroids having 20 ng of desogestrel and 10 ng of EE kicked the expression of LDLR and SREBP2 to a much higher level, and the changes of their expression profile were comparable with all contraceptive steroid combinations. Since it was already known that activated SREBP2 [40,41] was responsible for transcribing LDLR, more expression of SREBP2 was expected to provide more activated protein to transcribe more LDLR. Eventually, this was the effect we found in this study that the combination OC induced parallel increase in the expressions of SREBP2 and LDLR by the differentiated placental trophoblast cells from human origin.

Since the primary cultures from time to time were made with placentae obtained from different donors and as the chances of variations are expected from one culture to another because they were made from different sources, we rechecked the above phenomenon with cells obtained from a stable placental trophoblast cell line culture (JAR cell line). In a similar study with JAR cell line by Western blot, i.e., keeping one contraceptive steroid concentration constant and to vary the other, comparable results were obtained in the expression profiles of LDLR and SREBP2. In both cases, the expressions of LDLR and SREBP2 were compared, and it was found that there were similar trends in changing the expression profile of both entities with the changes in concentration of steroid components of the combination OC. Thus, the results from primary placental trophoblast cell culture and the culture of placental trophoblast cell line (JAR) showed the consistency of the same phenomenon — the combination OC steroids of 20 ng desogestrel and 10 ng EE stimulated both LDLR and SREBP2 in comparable manner.

Finally, we were interested to see whether the combination-OC-stimulated highly expressed LDLR was also efficient for faster clearance of extracellular LDL particles. When we compared the profile of LDL uptake in the presence and absence of the OC of interest, we found more uptake of LDL (fluorogenic Dil-LDL was used for quantitative estimation) from the medium in the presence of combination OC only. The experiment was done with the cells obtained from JAR cell culture.

Therefore, the outcome of the present study reveals that the combination OC with a composition of 20 ng desogestrel and 10 ng EE was responsible to maintain a high-profile expression of LDLR through the stimulation of its transcription factor SREBP2 and at the same time was able to keep the receptors highly efficient in their functional activity. Therefore, the combination of 20 ng desogestrel with 10 ng EE in a combination OC plays a potential role on plasma LDL clearance and hence may help in reducing atherogenic exudation.

Acknowledgment

The authors thank Dr. K.K. Roy, Professor of Obstetrics and Gynaecology, AIIMS, New Delhi, India, for providing human placenta.

References

[1] Diamant YZ, Neuman S, Shafrir E. Effect of chorionic gonadotropin, triamcinolone, progesterone and estrogen on enzymes of placenta and liver in rats. Biochim Biophys Acta 1975;385:257–67.
[2] Crews JK, Khalil RA. Antagonistic effects of 17b-estradiol, progesterone and testosterone on Ca21. Entry mechanisms of coronary vasoconstriction. Arterioscler Thromb Vasc Biol 1999;19:1034–40.
[3] Christodoulakos GE, Lambrinoudaki IV, Economou EV, et al. Differential effect of hormone therapy and tibolone on lipids, lipoproteins and the atherogenic index of plasma. J Cardiovasc Pharm 2006;47:542–8.
[4] Shufelt L, Merz NB. Contraceptive hormone use and cardiovascular disease. J disease J Am Coll Cardiol 2009;53:221–31.
[5] Barbieri RL, Speroff L, Walker AM, Mcpherson K. Therapeutic controversy. The safety of third generation oral contraceptives. J Clin Endocr Metab 1999;84:1822–9.
[6] Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986;232:34–47.
[7] Goldstein JL, Brown MS. The LDL receptor locus and the genetics of familial hypercholesterolaemia. Ann Rev Genetics 1979;13:259–89.
[8] Goldstein JL, Brown MS. The low density lipoprotein pathway and its relation to atherosclerosis. Ann Rev Biochem 1979;46:897–930.
[9] Podrez EA, Febbraio M, Sheibani N, et al. Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. J Clin Invest 2000;105:1095–108.
[10] Kume N, Murase T, Moriwaki H, et al. Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. Circ Res 1998;83:322–7.

[11] Adachi H, Tsujimoto M, Arai H, Inoue K. Expression cloning of a novel scavenger receptor from human endothelial cells. J Biol Chem 1997;272:31217–20.
[12] Chen WJ, Goldstein JL, Brown MS. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit mediated internalization of the low density lipoprotein receptor. J Biol Chem 1990;265: 3116–23.
[13] Bansal A, Gierach LM. The NPXY internalization signal of the LDL receptor adopts a reverse turn conformation. Cell 1991;67:1195–201.
[14] Davis CG, van Driel IR, Russell DW, Brown MS, Goldstein JL. The low density lipoprotein receptor. Identification of amino acids required for rapid endocytosis. J Biol Chem 1987;262:4075–82.
[15] Ramakrishnan G, Rana A, Das C, Chandra NC. Study of low-density lipoprotein receptor regulation by oral (steroid) contraceptives: desogestrel, levonorgestrel and ethinyl estradiol in JEG-3 cell line and placental tissue. Contraception 2007;76:297–305.
[16] Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 1997;89:331–40.
[17] Shea-Eaton WK, Trinidad MJ, Lopez D, Nackley A, McLean MP. Sterol regulatory element binding protein-1a regulation of the steroidogenic acute regulatory protein gene. Endocrinology 2001; 142:1525–33.
[18] Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss III JF. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 1986;118:1567–82.
[19] Hunt T, Geetha R, Warga E. The bioavailability of desogestrel/ethinyl estradiol tablets relative to the oral solution. Clin Drug Invest 1998;15: 507–14.
[20] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[21] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4.
[22] Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955;34:1345–53.
[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[24] Stephan ZF, Yurachek EC. Rapid fluorometric assay of LDL receptor activity by Dil-labelled LDL. J Lipid Res 1993;34:325–30.
[25] Brown MS, Goldstein JL. A proteolytic pathway that controls the cholesterol content of membranes, cells and blood. Proc Natl Acad Sci USA 1999;96:11041–8.
[26] Brown MS, Goldstein JL. Cholesterol feedback: from Schoenheimer's bottle to SCAP's MELADL. J Lipid Res 2009:S15–27.
[27] Stadel BV. Oral contraceptives and cardiovascular disease (first of two parts). N Engl J Med 1981;305:612–8.
[28] Stadel BV. Oral contraceptives and cardiovascular disease (second of two parts). N Engl J Med 1981;305:672–7.
[29] Royal College of General Practitioners' Oral Contraceptive Study. Mortality among oral contraceptive users. Lancet 1977;2:727–31.
[30] Dalen JE, Hickler RB. Oral contraceptives and cardiovascular disease. Am Heart J 1981;101:626–39.
[31] Meade TW, Greenberg G, Thompson SG. Progestogens and cardiovascular reactions associated with oral contraceptives and a comparison of the safety of 50- and 30-μg preparations. Br Med J 1980;280:1157–61.
[32] Foulon T, Payen N, Laporte F, et al. Effects of two low-dose oral contraceptives containing ethinylestradiol and either desogestrel or levonorgestrel on serum lipids and lipoproteins with particular regard to LDL size. Contraception 2001;64:11–6.
[33] Barkfeldt J, Virkkunen A, Dieben T. The effects of two progestogen-only pills containing either desogestrel (75 μg/day) or levonorgestrel (30 μg/day) on lipid metabolism. Contraception 2001;64:295–9.
[34] Endrikat J, Klipping C, Gerlinger C, et al. A double-blind comparative study of the effects of a 23-day oral contraceptive regimen with 20 μg ethinyl estradiol and 75 μg gestodene and a 21-day regimen with 30 μg ethinyl estradiol and 75 μg gestodene on hemostatic variables, lipids and carbohydrate metabolism. Contraception 2001;64:235–41.
[35] Ross R. Cell biology of atherosclerosis. Ann Rev Physiol 1995;57: 791–804.
[36] Ging Y. Sterol-regulates ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake. Cell Metabol 2006;3:15–24.
[37] Hua X, Yokoyama C, Wu J, et al. SREBP-2, a second basic helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. Proc Nat Acad Sci USA 1993;90:11603–7.
[38] Eberle D, Hegarty B, Bossard P, Ferré P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. Biochemie 2004;86:839–48.
[39] Ramakrishnan G, Chandra NC. Estradiol regulates insulin dependent stimulation of LDL-receptor expression in HepG2 cells. Indian J Clin Biochem 2006;21:8–14.
[40] Briggs MR, Yokoyama C, Wang X, Brown MS, Goldstein JL. Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. Identification of the protein and delineation of its target nucleotide sequence. J Biol Chem 1993;268:14490–6.
[41] Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H. Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice over producing sterol regulatory element binding protein-2. J Clin Invest 1998;101:2331–9.