Estradiol Regulates Insulin Dependent Stimulation of LDL-Receptor Expression in HepG₂ Cells

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ABSTRACT

The low density lipoprotein, one of the major vehicle for extraneous cholesterol, internalizes into the cells through the process of LDL-receptor mediated endocytosis. The expression of LDL-receptor on the cell surface is a function of various hormone regulated transcription of the receptor gene. The present study elucidates the differential expression pattern of LDL-receptor protein in human hepatoma HepG₂ cells by the influence of two hormones, insulin and estrogen (β -estradiol), as compared to the basal level expression of the receptor protein. The combined effect of insulin and β -estradiol reveals that β -estradiol is the ultimate regulator between these two hormones and supershades the message of insulin on LDL-receptor expression. The receptor protein level immunoblotted by anti LDL-receptor antibody after treating cells with insulin, β -estradiol and mixture of both also reflects the same phenomena. This comparative study makes it consistent that cell saturation with sterol (β -estradiol) is the prime regulator of LDL-receptor expression between the two hormones, insulin and estrogen.

KEYWORDS

LDL-receptor, Estrogen, Insulin, HepG₂ cells, Cholesterol.

INTRODUCTION

The low density lipoprotein (LDL) receptor is a widely distributed cellular endocytic receptor that functions to internalize cholesterol containing LDL particles (1, 2). The internalized cholesterol is used for cell growth and new plasma membrane synthesis (3, 4). The cell surface expression of LDL-receptor protein is a reflection of the rate of transcription of low density lipoprotein receptor gene, which is also found regulated by intracellular cholesterol concentration, hormones and growth factors (5-9). It is reported that the LDL promoter in cell nucleus contains a steroid response element (SRE). Binding of SRE by steroid response element binding protein (SREBP) makes the LDL-receptor gene transcriptionally active. One endoplasmic reticulum (ER) membrane bound protease, SREBP cleavage activating protein (SCAP), generates functionally active transcription factor SREBP-2 from SREBP precursors. SREBP-2 binds

SRE region on LDL-promoter in the nucleus favoring the following recruitment of a histone acetyl transferase (P/CAE), general transcriptional activator (CBP/ p300) and other proteins to switch on LDL-receptor gene transcription. The SCAP remains inactivated in sterol saturated cells and gets activated with depletion of cellular sterol concentration (10, 11). Although many hormones and growth factors have shown (5-8) stimulatory effect on LDL-receptor protein transcription, it is not yet lucid whether those hormones and growth factors have their independent role on the stimulatory mechanism of LDLR gene or they are encompassed by the sterol regulatory pathway of LDLR gene regulation. Inquisition in understanding this unsolved arena, led us to run this comparative study to evaluate the interdependency of two hormones, Insulin and Estrogen(β -Estradiol) on LDLR expression in HepG₂ cells. Our study shows the dominance of β -Estradiol over Insulin for expressing LDLR gene, suggesting the probability of regulatory control of estrogen directed SRE regulatory pathway on insulin executed changes towards LDLR expression.

MATERIALS AND METHODS

Chemicals : Insulin, β -estradiol, DMEM-powder, fetal calf serum (FCS), antibiotic antimycotic solution (100x)

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and HEPES were purchased from Sigma Chemical Co., St. Louis, MO, USA. LDL receptor goat polyclonal primary antibody was obtained from Santa Cruz Biotechnology, Inc. California, USA (DAKO LSAB + Kit), peroxidase package was from DAKO corporation, CA, USA. Anti goat HRP conjugated secondary antibody, protean nitrocellulose membranes and femtolucent detection Kit were purchased from Genotech, St. Louis, MO, USA. Plastic wares for cell-culture were from Becton Dickinson Labware, New Jersey, USA. All other chemicals used were of analytical-reagent grade.

Cell culture : HepG₂ cells were a gift from Dr. Vijay Kumar, ICGEB, New Delhi, India. Cells were grown in DMEM containing glucose (4.5 g/litre), HEPES (5.96 g/litre), NaHCO₃ (3.7 g/litre) and 10% FCS (v/v). Penicillin (100 units/ml), streptomycin (100 mg/ml) and amphotericin B (250 ng/ml) were included in the cell cultures, which were carried out at 37°C in an atmosphere of 95 % air and 5% CO₂, either on 90 mm dishes or 25 cm² flasks.

Hormones : β -estradiol powder was initially dissolved in minimal quantity of 50% ethanol (v/v) followed by dilution with distilled water to the respective concentrations used directly in the medium. An insulin solution (10 mg/ml in 25 mM HEPES, pH 8.2) was diluted in DMEM to 10³ U/ml and required quantity of this preparation was used to attain the respective insulin concentration in the medium.

Immunocytochemistry and hormone challenge : HepG₂ cells that reached approximately 90% confluency on 12 mm microscopic cover glass equipped in a medium-full culture plate, were washed twice in 0.01M PBS. Fresh 2 ml of DMEM was added with and without hormones, insulin or β -estradiol or mixture of two, in various doses (see the result) and incubated for 2 hrs at 37°C on a rocker platform under constant shaking. The concentrations of the hormones were within the cytotoxic limits so that there was no cell death in 2hr incubation period. The medium was then removed and the cells were washed twice with 0.01M PBS. Cells were fixed in absolute acetone for 10 min at 4°C. Endogeneous peroxidase was quenched by treating the cells with 4% H₂O₂ in methanol for 30 min at room temperature followed by rinsing in 1M Tris-HCl, pH 7.2 containing 0.01% Triton-X 100 (buffer-A) for 5 min thrice. Non-specific binding was blocked by placing the cover glasses with 5% non-fat milk in PBS for 1 hour at room temperature. Two washes were repeated with buffer-A. This was followed by overnight incubation in a humidified chamber with specific primary antibody against LDL receptor at a dilution of 1:50 in 0.01M PBS at 4°C. Pre-immune serum was also used as a control to observe any nonspecific interaction with the receptor protein. The cells were washed thrice in buffer-A

followed by incubation with biotinylated secondary antibody for 1 hr at room temperature. The cells were then incubated for 1 hr with avidin-HRP after a 3x-wash in buffer-A, washed again in buffer-A and treated with DAB buffer (0.06% DAB, 50 mM Tris-HCl, pH 7.6, 1% H₂O₂) for 3-5 min at room temperature for visualisation of antigen-antibody complex. Finally, cover glasses were rinsed in distilled water, counter stained with Mayer's haematoxyline for 1min, excess stain rinsed with water, air-dried, dehydrated in xylene for 5 min and mounted on using DPX mountant. Photographs were taken after observation at 20X magnification by a Nikon DXM 1200 digital camera attached to a Nikon microphot FXA microscope.

Preparation of HepG₂ cell lysate: Cells were grown approximately to 90% confluency in 100 mm culture plates. The medium was removed from each plate, washed with 0.01M PBS and 5ml of fresh DMEM was added with and without hormone(s) in respective doses (see the result). The cells were then incubated at 37°C in presence of 95% air and 5% CO₂ for 2 hr on a rocker and subsequently washed with 0.01M PBS twice and finally scraped off in 2 ml of the same PBS. The cell pellets were collected after centrifugation at 4°C, 1000 g for 15 min. and then lysed in 400 μ l of lysis buffer, containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 0.5% Triton-X 100, 5 mM EDTA with 2 mM PMSF and 10 U/ml aprotinin added just before use, by vortexing strongly till the consistency of the solution was changed. This lysed suspension was kept on ice for 30 min and then spun at 17,000 g for 15 min at 4°C. The supernatant was collected and protein content was estimated.

Protein estimation: Protein estimation was according to the method of Bradford *et al.* using bovine serum albumin as the standard (12).

SDS-PAGE and Immunoblotting: Electrophoresis was carried out on 10% polyacrylamide gels (1.5 mm thick), overlaid by a 4.0% stacking gel, in SDS according to the procedure of Laemmli (23). The protein bands were visualized by the Coomassie or silver stains.

For immunological identification, the proteins were transferred onto nitrocellulose sheets (24). The sheets were washed with blocking buffer containing 5% milk powder in 0.01M PBS for 1 h followed by 4 or 5 washes in 0.01M PBS, 1% bovine serum albumin containing 0.05% Tween 20, and then they were incubated for 1 h at room temperature with a 200-fold diluted normal fetal calf serum. Subsequent to further washes with the aforesaid wash-buffer, incubation of the sheets with diluted antiserum (LDLR goat polyclonal primary antibody, 1:500 dilution) was carried out for 2 h at room temperature. Bound antibody was treated with an anti-goat-HRP conjugated

secondary antibody and then detected by femtoLUCENT detection kit (Genotech, St.Louis,MO, USA).

RESULTS

Immunocytochemistry in Fig. 1 has shown the basal level expression of LDLR protein in normal HepG₂ cells treated with anti-LDLR antibody in absence of either insulin or estrogen (β -estradiol).

Pre-incubation of HepG₂ cells with varying concentrations of insulin gave an optimal LDLR

Figure 1. Immunocytochemical expression of LDL receptor protein in normal HepG₂ cells. The receptor protein was treated with anti-LDLR (see material and methods) antibody in absence of either insulin or estradiol. X20

expression at 50 U per ml culture medium of insulin concentration (normal human serum level varies between 4-25 U per ml) (Fig. 2). Increase of insulin level upto 100 U per ml medium (not shown) did not show any further change on LDLR expression. The downstream signaling mechanism is not very clearly known for such LDLR expression occurred by insulin. The biological activity of this commercial preparation of insulin may be inferior to the *in vivo* activity found in human serum. This may be the reason of having insulin concentration (50 U per ml) needed beyond the normal human serum level (4-25 U per ml) to show optimal LDLR expression in HepG₂ cells.

Pre-incubation of HepG₂ cells with β -estradiol had shown the optimal expression of receptor protein at a hormone concentration of 50 ng per ml culture medium (Fig. 3). No apparent change in the level of receptor expression was observed up to 100 ng per ml culture medium of hormone concentration and henceforth the expression density started falling off and reached to the basal expression level at the concentration of 150 ng hormone per ml culture medium. Although normal serum estrogen concentration per ml exists in pico gram level; such a high concentration required as external source of steroid in culture medium of this *in vitro* experiment for optimal expression of receptor protein might be the fact to attain the optimal intracellular sterol concentration (estradiol) to trigger the receptor gene expression, which correlates with the sterol regulatory mechanism of LDL receptor transcription regulation. A dose as high as 150 ng of estradiol per ml culture medium was used just to see the relative requirement of extracellular sterol to raise intracellular sterol level to a height that brings down the optimal receptor expression back to basal level following the intracellular sterol regulatory of mechanism over LDLR gene expression (10, 18).

The combined effect of β -estradiol and Insulin on LDLR expression, reflected the dominance of β -estradiol effect (Fig. 4). 50 U per ml culture medium of insulin was used with each of three different concentrations of β -estradiol (10, 75 and 150 ng per ml culture medium) separately. The combined effects of hormones on LDLR expression in each combination were compared with the single effect of each of the hormones of equivalent concentration (Fig. 4, panel a,d; b,e; c,f; and Fig. 2, panel c). In each case the mixed effect of expression was comparable with the corresponding concentrations of β -estradiol shown in panels a,b,c of Fig. 4. The stimulatory effect of insulin concentration on LDLR expression shown in Fig. 2 was missed in presence of β -estradiol and only the effect of the amount of β -estradiol used in

Figure 2. Immunocytochemical expression of LDL receptor protein with varying concentration of insulin [5 U (a), 25 U (b) and 50 U (c)] per ml culture medium. Panel- c shows optimal expression. X20

Figure 3. Immunocytochemical expression of LDL receptor protein with varying concentration of β -estradiol [2 ng (a), 20 ng (b), 50 ng (c), 100 ng (d) and 150 ng (e)] per ml culture medium. Panel-c and -d show optimal expression. X20

Figure 4. Comparison of immunocytochemical expression of LDL receptor protein with varying concentration of β -estradiol in presence (panel d, e, f) and absence (panel a, b, c) of insulin. Concentrations of β -estradiol per ml medium used were 10 ng (a), 75 ng (b), 150 ng (c) respectively. Effect of 50 U insulin used per ml medium with each respective concentration of β -estradiol are shown in panels d, e and f respectively. Comparable expressions are observed in panels a, d; b, e; and c, f. No significant effect of insulin on β -estradiol is apparent in this comparative study. X20

each mixture got reflected in the intensity of LDLR expressions. The induced expression from mixture of hormones was consistently comparable with the parallel β -estradiol induced expression of LDLR protein. Such an effect raised the idea that β -estradiol was probably acted as a parent regulator in the process of insulin induced regulatory mechanism for LDLR protein expression.

To find the effect of β -estradiol more consistently on insulin induced expression profile of LDLR protein, varying concentrations of insulin (0, 50 and 100 U per ml culture medium, panel a, b & c respectively) were used with a fixed concentration of β -estradiol (25 ng β -estradiol per ml culture medium) (Fig. 5). 25 ng β -estradiol per ml culture medium itself showed approximately 50 percent intensity of optimal expression shown in Fig. 3. There was no remarkable change in expression intensity with varying insulin concentrations; rather in each case, the apparent

expression intensity was comparable with the single concentration of β -estradiol (25 ng) in the culture medium of HepG₂ cells (Fig. 5, panel a).

The dominance of estrogen upon insulin induced expression of LDLR protein in HepG₂ cells was also reproduced consistently in the immunoblot of HepG₂ cell extracts after hormone treatment (single and mixture) (Fig. 6). It is apparent that the stimulatory effect of 50 U insulin per ml culture medium (lane 3 as compared to lane 2) remained dormant (lanes 5 and 7) in presence of 50 ng and 150 ng of β -estradiol per ml medium. The intensity of expression with 50 ng (optimal expression) and 150 ng (same as zero level as shown in lane-1) of β -estradiol per ml medium are shown separately in lanes 4 and 6 respectively. Hence the effect of β -estradiol (50 ng and 150 ng per ml medium) became dominant (Fig. 6, lanes 4, 6) superseding the effect of insulin (Fig. 6, lanes 5, 7) on LDLR protein expression in HepG₂ cells.

1. Control, 2. Insulin 5 U, 3. Insulin 50 U, 4. Estradiol 50 ng, 5. Insulin 50 U + Estradiol 50 ng, 6. Estradiol 150 ng, 7. Insulin 50 U + Estradiol 150 ng, per ml culture medium.

Figure 6. Analysis of expression of LDL receptor protein from the immunoblot of receptor with anti-LDLR antibody in presence of insulin and estradiol, alone and in combination, in the culture medium. The apparent expressions were relatively dependent on β -estradiol concentration when used in combination with insulin. Lanes 5 and 7 are comparable with lanes 4 and 6.

DISCUSSION

Through the past decade, a growing consensus that cells probably control their membrane composition by sensing sterol / cholesterol directly rather than by monitoring a physical property of the membrane, such as thickness or fluidity (13), has developed directional interests to study various aspects in cellular cholesterol homeostasis. LDL-receptor (LDLR) is one of the gateways that permit extra cellular cholesterol to enter into cells by endocytosis. Many hormones and growth factors (5, 8, 14, 15) regulate the transcription of gene for the LDL-receptor (LDLR) protein. Recent research (16, 17) reports that depletion of cholesterol from cells enhances the transcription machinery, whereas replenishment with cholesterol down regulates the process. It is also reported that sterols in general mimic similar effect (18). On the contrary evidences encounter that administration of hormones like 17α -ethinyl estradiol (19) and β -estradiol (20, 21) in pharmacologic doses elicited an increase in the number of lipoprotein receptors on cellular membrane of respective tissues. These pharmacologic doses were not enough to saturate the cells with sterol. Ironically cells get saturated with cholesterol (the precursor molecule for sterol in sterol synthesizing cells) in sterol saturated state of respective cells. Though all those studies independently have shown their own influence on the rate of transcription of LDLR gene, no comparative study was aimed to examine the interdependency between those extraneous factors e.g. hormones or growth factors. In the present study we have compared two hormones, insulin and β -estradiol, distinct in their aqueous solubility for rating the degree of expression of LDLR protein in HepG₂ cells. Insulin is aqueous soluble and nurses the signaling mechanism of LDLR expression remaining by itself on the exterior zone of membrane, whereas β -estradiol being membrane soluble can diffuse through plasma membrane to saturate intracellular cytoplasm in addition to its interaction on LDLR expression at the level of plasma membrane. No downstream signaling mechanism is known for either of these two hormones (insulin and β -estradiol) for their interaction with LDL receptor protein at the plasma membrane level. A sterol regulatory mechanism for LDL receptor protein transcription is slowly emerging out (10, 18) that only partially satisfies the effect of β -estradiol on receptor protein expression. The mechanism of insulin dependent stimulation of LDL receptor yet remains to be explored.

The Fig. 4 in current study summarizes the fact that in each respective combination (panel d, e and f) of hormones, β -estradiol superseded the stimulatory effect of insulin (insulin stimulation is documented separately in Fig. 2) on LDLR expression. The intensity of LDLR expression in each event of mixed hormone

was comparable to that found with β -estradiol when applied alone in a concentration similar to that used in mixture (panel a , b and c in Fig. 4). The basal level of expression of LDLR in HepG₂ cells is shown in Fig. 1.

50-100 ng of β -estradiol per ml culture medium maintained the optimal expression level of receptor (panel c and d in Fig. 3). The receptor expression got maximally inhibited at 150 ng β -estradiol per ml. culture medium (panel e Fig. 3). Both these effects were apparent in the receptor expression activity of mixed hormones having 75 ng and 150 ng β -estradiol component separately with 50 U insulin respectively (Fig. 4, panel b, e and c, f). Adding increasing dose of insulin (50 - 100 U per ml culture medium) with a given concentration of β -estradiol (25 ng per ml culture medium) even did not show any stimulatory effect of insulin over the effect with respective concentration of β -estradiol. All these cytochemical experiments thus have developed the view that β -estradiol modulated LDLR expression deserves priority over insulin induced expression of LDLR protein.

The out-comings for LDLR expression regulation from above experiments were consistently reproduced with transblotted receptor protein from cell lysate and identified by anti-LDLR antibody after various treatment with aforesaid hormones (single and mixture) with stimulatory and inhibitory concentrations. The expression of protein in lane 5 and lane 7 on the immunoblot (Fig. 6) represented intensity equivalent to that of the lanes 4 and 6. The expression density of receptor protein by 50 U of insulin per ml culture medium, shown in lane 3 (Fig. 6), has probably been encompassed in lane 5 and 7 by the intrinsic signaling mechanism of steroids reported elsewhere (22). The β -estradiol (estrogen) at two distinct concentrations (50 and 150 ng) have extremely opposite effects on the magnitude of LDLR protein expression in HepG₂ cells. The overall results from these experiments thus emphasizes the fact that in the events of insulin and estrogen, the latter (here β -estradiol) got the priority in the regulation of LDL-receptor (LDLR) protein expression.

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