ISSN: 2279 - 0594

Journal of Biomedical and Pharmaceutical Research

Available Online at www.jbpr.in

CODEN: - JBPRAU (Source: - American Chemical Society)

Volume 4, Issue 5: September-October: 2015, 01-09

Research Article

Effect of Insulin on Expression of LDLR, SREBP-2 and Cell Proliferation in Chang (normal) Liver Cells

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Received 10 September 2015; Accepted 02 October 2015

ABSTRACT

Low-density lipoprotein receptor (LDLR) maintains lipid metabolism and its expression is regulated by Sterol Regulatory Element Binding proteins (SREBPs). This study was designed to investigate the effect of insulin, a mitogen on LDLR, SREBP-2 receptor expression and cell proliferation.

Insulin was found to have a stimulatory effect on the expression of LDLR and its transcription factor SREBP-2. Fluorescent dye (Dil) labelling assay revealed significantly increased uptake of Dil-LDL in the presence of insulin. Insulin also showed increased rate of cell replication. These findings suggested that LDLR expression is enhanced in the presence of insulin, which in turn results in increased uptake of LDL into the cells causing increased cell proliferation.

The elevated expression of SREBP-2 indicated the influence of insulin on the transcription of genes responsible for increased expression and activity of LDLR.

Keywords: LDL receptor, SREBP-2, Atherosclerosis, LDL uptake and BrdU Assay.

INTRODUCTION

Cholesterol homeostasis in mammalian cells is maintained by a feedback regulatory system that senses cellular cholesterol content and appropriately modulates the transcription of genes of the components of cholesterol metabolism [1, 2]. The low-density lipoprotein receptor (LDLR), a type-I trans-membrane glycoprotein, is well known for its involvement in clearing cholesterol-laden LDL particles from blood vessels [1, 3, 4-6]. The expression of LDLR gene is regulated by a family of endoplasmic reticulum (ER)-bound transcription factors called Sterol Regulatory Element Binding proteins (SREBPs) [1, 2].

Oxidative stress leads to modification of LDL into its oxidized counterpart, oxidized LDL (ox-LDL), which gets accumulated in the blood vessels becoming an agent for a major cause of atherosclerosis and coronary artery disease [7]. Inactive LDLR or its low-level expression results in accumulation of LDL particles in blood vessels. On the other hand, the receptor of ox-LDL, a lectin-like oxidized LDL receptor (LOX-1) binds ox-LDL and acts as a signal generator for the formation of pro-atherogenic components in blood vessels. Formation of pro-atherogens acts as a stimulus for atherothrombosis and coronary artery disorders [5]. Insulin is known to regulate multiple biological activities [8-12], resulting in enhanced glucose transport and maintenance of adequate blood glucose levels [13-16]. Insulin resistance is also associated with LDL accumulation in blood vessels as a result of poor clearance by functionally impaired LDLR [16, 17].

There are reports that in cancer cells the feedback regulation of cholesterol is lost [18]. It is not known whether LDL cholesterol has any role over cell proliferation in tumor-formation. It has also been reported that LDL receptor activity in HepG2 cells is hormonally regulated; insulin increases LDLR activity [19-21]. In Diabetes mellitus, when the body is unable to produce or become resistant to endogenous insulin, LDLR activity gets impaired [22]. The increasing utilization of insulin has become a cause for concern because findings from several observational trials have suggested an association with an increased risk of developing cancer [23]. Evidence for hormone-induced

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mitogenicity appears to be most prevalent in prostate, breast, pancreatic, and colorectal cancers [23].

This study, therefore, has been aimed at comparing the feedback regulation of intracellular cholesterol homeostasis and its role on cell proliferation in the presence and absence of the mitogen, i.e. insulin.

MATERIALS AND METHODS

Chemicals

Eagle's Minimum Essential Medium (EMEM) Nutrient mixture was obtained from GibcoBRL, New York, NY, USA. Fetal calf serum (FCS), antibiotic antimycotic solution (100X), acrylamide/ bis-acrylamide, insulin and HEPES were purchased from Sigma Chemical Co., St. Louis, MO, USA. Coomassie brilliant blue R-250 was purchased from Bio-Rad laboratories, Hercules, USA. Estimation was done in Shimadzu UV-VIS spectrophotometer, Shimadzu Corporation, Kyoto, Japan. Low-density lipoprotein receptor LDLR goat polyclonal primary antibody (N-17, sc-11822) epitope at the N-terminus of LDLR of human origin) and SREBP-2 goat polyclonal primary antibody ((N-19, sc-8151) were obtained from Biotechnology Inc., Santa Cruz, CA, USA. Anti-goat and Anti-mouse HRP-conjugated secondary antibody were procured from Inc., Santa Cruz, CA, USA. Endogenous control GAPDH mouse monoclonal antibody for western blotting and Western blotting ECL reagent (Enhanced Chemi Luminescence) Luminol were obtained from Santacruz Biotechnologies, CA, USA.

Nitrocellulose membrane (0.2 µm pore size) was purchased from Whatmann GmbH, Dassel, Germany and Bovine Serum Albumin fraction V (BSA), NaCl-KBr salt solution, 100% trichloroacetic acid, o-phthalaldehyde, cholesterol standard were obtained from Sigma, St. Louis, USA. Fresh human plasma for LDL isolation was obtained from the Blood Bank, All India Institute of Medical Sciences. Dil was purchased from Sigma, St Louis, USA and its estimation was carried out in a Bio-Rad spectroflourimeter (Bio-Rad Technologies, USA). BrdU kit was procured from Calbiochem. All plastic ware were obtained from Iwaki, Japan and all glass wares were procured from Borosil, Mumbai, India. Deionised water used for preparation of media, buffers and other reagents was obtained through Millipore water filtration systems, and milli-Q water was used for preparation of all buffers. All other chemicals used for this study were of analytical reagent grade. Human normal hepatoma cell line *Chang* was obtained from NCCS, Ganeshkind, Pune, India.

Cell culture

Chang liver cells were grown in Eagle's Minimum Essential Medium (EMEM) Nutrient mixture (SIGMA) containing HEPES (5.96g/ml), $NaHCO_3$ (3.7 g/ml) supplemented with 10% FBS and 100 U/ml penicillin, 100µg/ml Streptomycin and 0.25 µg/ml Amphotericin-B. Cells were grown at 37^0C in a 95 % air – 5 % CO_2 environment. Cells were harvested in 0.025 % trypsin-2.5 mM EDTA in PBS and collected by centrifugation at 1000 x g for 10 minutes at Room Temperature (RT). The pellets were then suspended in the appropriate medium as per needs.

Cell lysate preparation

The cells were grown in 100 mm petri-plates or 75 cm^2 flask, and incubated in the presence or absence of different concentrations of insulin for 5 hours. These were then washed with 0.01 M ice cold PBS twice and then scraped off in 3 ml PBS. The cells were centrifuged at 1000 ×g for 10 min at 4°C and then lysed in 100 µl of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 0.5% (v/v) Triton X-100, 5 mM EDTA with 2 mM PMSF, 10 mcg/ml leupeptin and 10 U/ml aprotinin and by strong vortexing. The lysed suspension was kept on ice for 30 min and then spun at 12,000 rpm for 15 min at 4°C. The supernatant was collected and protein content was determined.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Electrophoresis was carried out on 10% polyacrylamide gels (1.5 mm thick), overlaid by a 4% stacking gel containing sodium dodecyl sulfate according to the standard procedure of Laemmli [24]. The proteins were transferred onto nitrocellulose membranes [25]. 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 three times in 10 mM PBS–0.1% Tween-20 for 10 min each. Anti-LDLR polyclonal primary antibody [Santa Cruz Biotechnology, Inc., C-7, sc-

11823] was added at a dilution of 1:3500 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 1:10000 dilution and kept for 2 h at room temperature followed by three washes in PBS–Tween buffer. For SREBP-2, polyclonal primary antibody dilution was 1:1000 and for secondary antibody dilution was 1:3000.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.

LDL isolation

LDL was prepared from human plasma by NaCl-KBr density gradient centrifugation according to Havel's method [26]. Dialysis was done in 0.01 M PBS for 24 hours changing buffer after 12 hours before use. Human blood was obtained from the Blood Bank of All India Institute of Medical Sciences through the clearance of the ethics committee of the institute.

Fluorescent (Dil) labeling of LDL

Low-density lipoprotein was mixed with Dil [30 mg of Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanin 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. After incubation, the density was raised from 1.006 to 1.063 by addition of NaCl–KBr salt solution as shown by Havel et al. [26]. The sample was then loaded into the tubes and layered with the equivalent-density salt solution and then centrifuged at 105,000×g for 22 h at 15°C. At the top of the tube the LDL fraction was collected and dialyzed similarly as shown by Havel et al. [26]. The quantification of Dil-LDL was done by Bradford's method based on total protein [27]. 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 [28].

Dil-LDL uptake study

Chang liver cells were seeded in the 12-well culture plates at cell density of 0.5×10^5 cells/well. The cells were incubated with varying concentrations of Dil-LDL (one concentration in one well and in triplicate) and with various concentration of insulin 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, following which the 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. Amount (ng/ml) of uptake was calculated from the estimated fluorescence of the Dil internalized into the cells.

Protein estimation

Protein estimation was done according to the Bradford method [27], using bovine serum albumin (BSA) as the standard.

Cell Proliferation Assay

Cell Proliferation was quantified using the Bromodeoxyuridine cell proliferation ELISA kit (QIA58, calbiochem). Chang liver cells were plated on 96 well plates at a concentration of $1x10^4$ cells/ well. The cells were then incubated with various concentrations of insulin for different time periods. Cell proliferation was analyzed by measuring the amount of BrdU incorporation as per protocol of BrdU ELISA kit (QIA58, calbiochem).

Cell counting

Chang cells were seeded in 24 well plates at a concentration of $1x10^4$ cells/ well. Then cells were treated with different concentrations of insulin for different time intervals. Cell counting was done in Z 2 coulter particle count and size analyzer – Beckman Coulter.

Statistical Analysis

All data was represented as mean ± SD. Unpaired Student's t-test (when two groups were involved) or one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison (when three or more groups were involved) was used to compare the mean values obtained from different sets of experiments. $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of insulin on the expression of LDL receptor and SREBP-2

In humans, the majority of the plasma cholesterol is cleared by LDL receptors (LDLR) [3]. It has been reported that insulin has a stimulatory effect on cellular expression of LDLR as well as its functional activity in HepG2 cells [29, 30]. Western blot assays with different doses of insulin were carried out to study the characteristic changes in LDLR and SREBP-2 expression in Chang cells with GAPDH as an internal control (Fig. 1).

Increasing concentrations of insulin up to 15 µU/ml showed significant increase in LDLR expression by Chang cells, which further increased more sharply at 25 µU/ml, as compared to control (without insulin). However, the LDLR expression regressed thereafter at 50µU/ml.

Similar effect of insulin was also observed on SREBP-2 expression which increased significantly with increasing concentrations of insulin in the medium up to 25µU/ml. Although thereafter, the activity of SREBP-2 decreased at 50 µU/ml concentration of insulin, but this activity was still significantly higher than the basal level expression in the control. These typical LDLR and SREBP-2 expression profiles may be due to the saturation of insulin titer at 25µU/ ml inside Chang cells

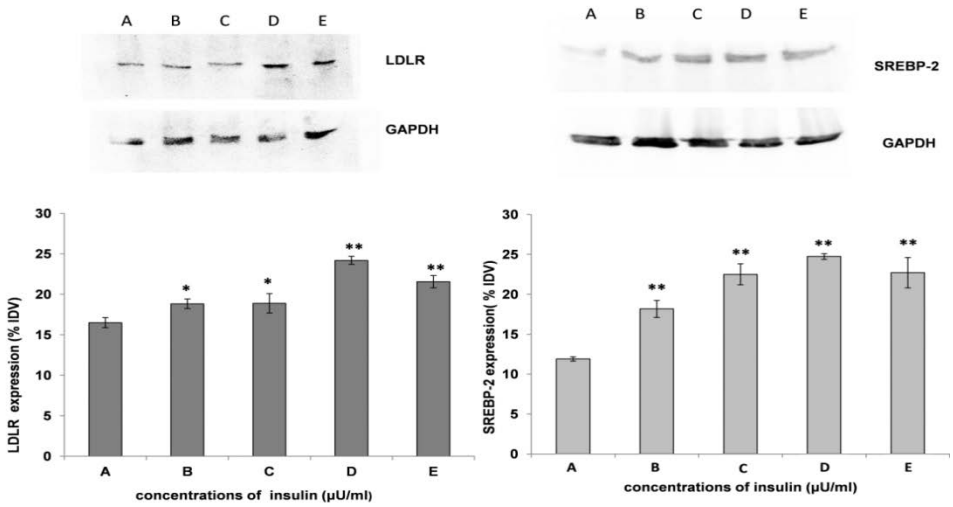

Figure 1: Effect of different concentrations of insulin on LDL receptor and SREBP-2 expression in Chang liver cells (% IDV). Concentration of insulin on X-axis (µU/ml): (A) 0, i.e. control, (B) 5, (C) 15, (D) 25, (E) 50.

*Statistics (for LDLR expression assay): One-way ANOVA followed by Dunnett's multiple comparison. *p* values against control: $p_5 < 0.05$; $p_{15} < 0.05$; $p_{25} < 0.01$; $p_{50} < 0.01$. % IDV was calculated as mean ± SD from three sets of experiments.

*Statistics (for SREBP-2 expression assay): One-way ANOVA followed by Dunnett's multiple comparison. *p* values against control: $p_5 < 0.01$; $p_{15} < 0.01$; $p_{25} < 0.01$; $p_{50} < 0.01$. % IDV was calculated as mean ± SD from three sets of experiments

Dil-LDL uptake in Chang liver cells

The effect of insulin on the functional activity of LDLR was analyzed fluorometrically by Dil-LDL uptake study in Chang Liver cell line. LDL was labelled with fluorescent dye Dil, which was used as a marker to detect the internalized LDL within the cell. Although, the uptake of extracellular LDL by Chang liver cell line culture was observed both in the presence and absence of insulin (control), but more utilisation of extracellular LDL by LDL

receptors was recorded in the presence of insulin as compared to the control (Fig. 2). The graph shows a steep uptake of Dil-LDL till 100 µU/ml insulin concentration, which attained saturation thereafter. This explains the fact that insulin increases functional activity of LDLR. These results suggested that insulin might have a role on LDL clearance from the blood circulation through cellular uptake by increased expression of LDL receptor on adjacent cell surface.

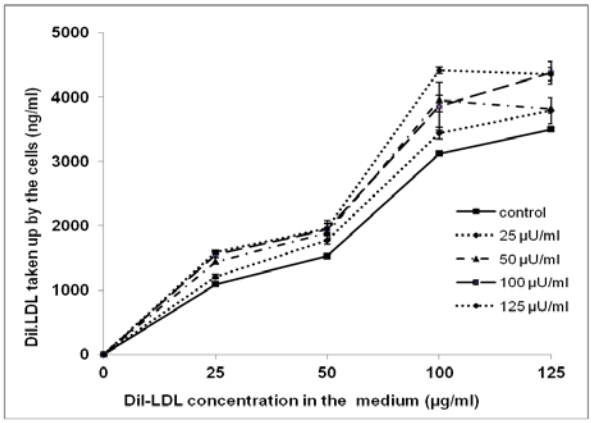

Figure 2: Dil-LDL uptake assay by Chang liver cells in presence of different concentrations of insulin after incubation for 5 h

Statistics: At different insulin concentration (25µU/ml), *p* values against control: p_{25} <0.05; p_{50} <0.001; p_{100} <0.01; p_{125}:ns; (50µU/ml), *p* values against control : p_{25} <0.001; p_{50} <0.05; p_{100} <0.01; p_{125} <0.01; (100µU/ml), *p* values against control : p_{25} <0.001; p_{50} <0.01; p_{100} <0.05; p_{125} <0.01; (125µU/ml),*p* values against control : p_{25} <0.0001; p_{50} <0.001;p_{100} <0.0001; p_{125} <0.001.

All values are mean ± SD (triplicate at each concentration). Statistical analysis was performed by Student's unpaired t-test.*p < 0.05, ** p< 0.01, ***p < 0.001

Cell proliferation assay

The role of insulin on the proliferation of Chang liver cells was further examined using BrdU-based cell proliferation ELISA (Fig. 3). No significant increase in the cell growth could be observed after 6 h incubation of Chang cells with 25 µU/ml insulin concentrations as compared to the control. However, the cell proliferation exhibited significant increase at 50 µU/ml and 100 µU/ml insulin concentrations after 6 h of insulin treatment. Significant cell increase in cell proliferation was observed at all concentrations of insulin with increasing time intervals of incubation.

The incorporation of BrdU into DNA implies increase in cell division; therefore, it is evident that insulin has a stimulatory effect in cell division turned cell proliferation in Chang liver cells. Since insulin has also shown to have a role on LDL uptake, it can be assumed that acquired LDL might also have a role in this cell proliferation. We, therefore, also checked the effect of LDL on cell proliferation using BrdU incorporation assay. Chang liver cells were incubated with 50 µg/ml and 100 µg/ml concentrations of LDL for different time intervals. A significant and consistent increase in cell growth was observed at regular intervals of 12, 24 and 48 h (Fig. 4). After 48 h, the growth phase saturated and there was no significant increase in cell growth thereafter. This explains the fact that insulin has a higher and prolonged mitogenic effect on cell proliferation.

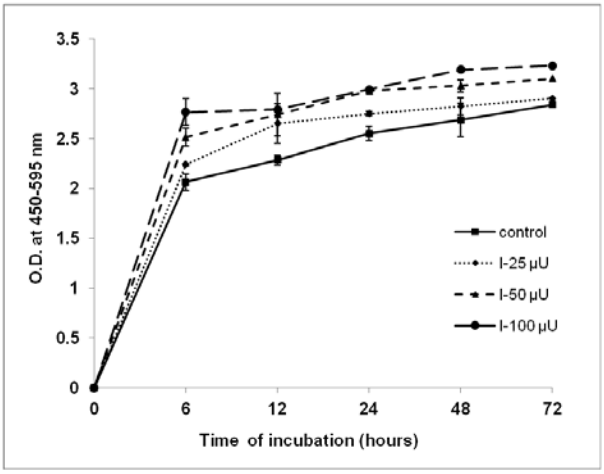

Figure 3: BrdUrd cell replication Assay in Chang liver cells in presence of different concentrations of insulin.

Statistics: *p* values: 6 hours against control: p_{25}: ns, $p_{50} < 0.001$; $p_{100} < 0.001$;12 hours against control: $p_{25} < 0.05$; $p_{50} < 0.05$; $p_{100} < 0.01$; 24 hours against control: $p_{25} < 0.001$; $p_{50} < 0.001$; $p_{100} < 0.001$;48 hours against control: p_{25}: ns; p_{50}: ns; $p_{100} < 0.01$; 72 hours against control: $p_{25} < 0.001$; $p_{50} < 0.001$; $p_{100} < 0.001$.All values are mean ± SD (triplicate at each concentration). Statistical analysis by one-way analysis of variance followed by Dunnett's multiple comparisons. $^{**}p < 0.01$, $^{***}p < 0.001$

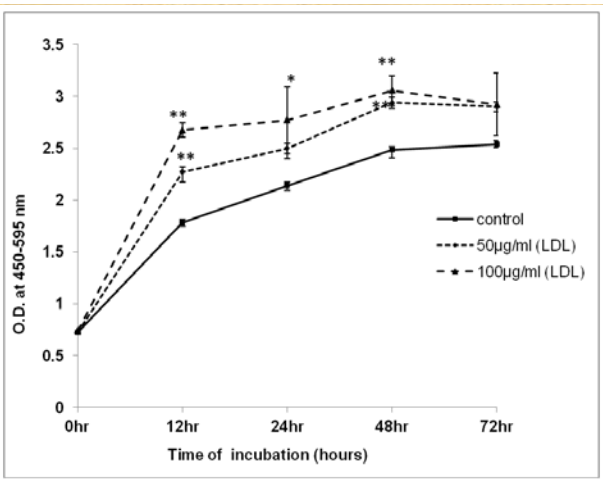

Figure 4: BrdU Assay in Chang liver cells in presence of LDL

Statistics: *p* values: 12 hours against control: $p_{50} < 0.01$; $p_{100} < 0.01$; 24 hours against control: p_{50}: ns; $p_{100} < 0.05$; 48 hours against control ,$p_{50} < 0.01$; $p_{100} < 0.01$; 72 hours against control: ns.All values are mean±SD (triplicate at each concentration). Statistical analysis by one-way analysis of variance followed by Dunnett's multiple comparison. $^{*}p < 0.05$, $^{**}p < 0.01$.

Cell counting

Effect of insulin and LDL on cell proliferation was also analyzed by cell counting. Chang liver cells were cultured in 24 well plates at a concentration of $1x10^4$ cells/ well. Cells were incubated with different doses of insulin and LDL; and were harvested at regular intervals of 12, 24, 48 and 72 h (Fig. 5). A significant increase in cell proliferation was observed in the presence of insulin, which was more pronounced in 100 µU insulin concentration than in 25 µU insulin concentration in the medium. Similar influence of LDL was observed with a significant increase in cell count in the presence of both 50 and 100 µg/ml LDL concentrations as compared to the control. Again, the influence of higher concentration of LDL on cell growth was evident as seen in case of insulin in the medium.

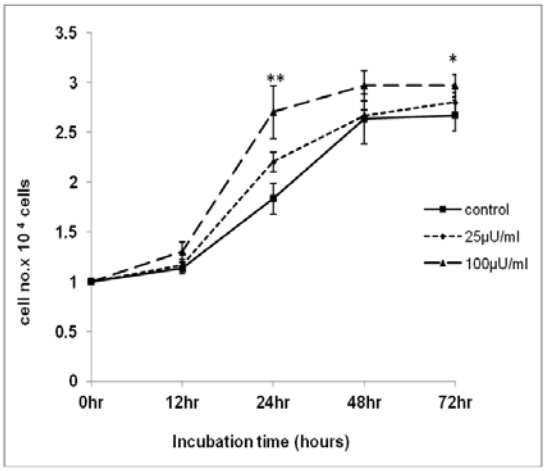

Figure 5: Cell counting in Chang liver cells after treatment with insulin.

Statistics: *p* values: 12 hours against control: p_{25}: ns; p_{100}: ns; 24 hours against control: p_{25}: ns; p_{100} < 0.01; 48 hours against control: p_{25}: ns; p_{100}: ns; 72 hours against control: p_{25}: ns; p_{100} < 0.05. All values are mean±SD (triplicate at each concentration). Statistical analysis by one-way analysis of variance followed by Dunnett's multiple comparison. *p <0.05, **p <0.01.

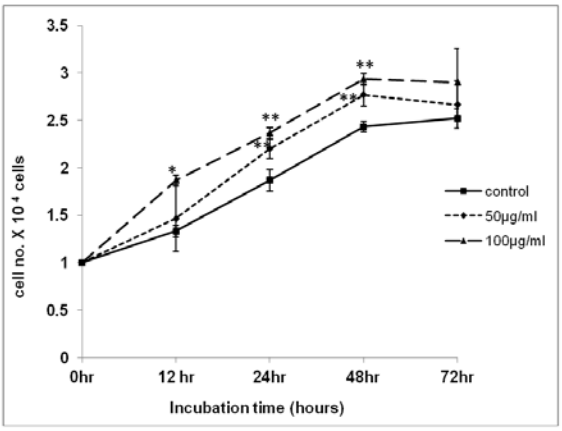

Figure 6: Cell counting in Chang liver cells after treatment with LDL.

Statistics: *p* values: 12 hours against control: p_{50}: ns; p_{100} < 0.05; 24 hours against control: p_{50}< 0.01; p_{100} <0.01; 48 hours against control: p_{50} <0.01; p_{100} <0.01; 72 hours against control: ns. All values are mean ± SD (triplicate at each concentration). Statistical analysis by one-way analysis of variance followed by Dunnett's multiple comparison. *p < 0.05, **p < 0.01.

DISCUSSION

Since LDLR gene expression is maintained by regulation of the endoplasmic reticulum-mediated transcription factor SREBP2-mediated feedback mechanism [1, 2, 31], we therefore compared the expression levels of LDLR and SREBP2 in Chang liver cells. Increased parallel expressions of LDLR as well as SREBP-2 were observed in the presence of insulin, which supports earlier findings that expression of LDLR is a posttranslational phenomenon of SREBP2 [1, 2, 32]. In previous studies Insulin has been found to stimulate cellular expression of LDLR as well as its functional activity in HepG2 cells [29,33]. When we compared the profile of LDL uptake in the presence and absence of the insulin in Chang cells, we found more uptake of LDL from the medium. This suggests that insulin has role in stimulation of functional activity of the LDL receptor.

BrdU and cell counting assays revealed significant increase in cell proliferation at all concentrations of insulin with increasing time intervals of incubation. Earlier studies have also reported that insulin stimulates the growth and proliferation of a variety of cells both in culture [34], and in vivo

[35]. The assay fortified the established mitogenic activity of insulin on cell proliferation. Similar assays with LDL also reported significant and consistent increase in cell growth at regular time intervals.

CONCLUSION

The data presented in this study suggests that insulin is responsible for the regulation and stimulation of LDL clearance by increasing the expression of LDLR and SREBP-2 in Chang liver cells. With the backdrop of the understanding that LDLR activity is controlled by a feedback regulatory system [3], this study has also shown that LDL enforces cell proliferation. It was further demonstrated that insulin too stimulates cell proliferation. Therefore, insulin induced cellular uptake of cholesterol, preferably in the form of LDL uptake, could be a mechanism of cell proliferation particularly in clinical pathology. This fortifies the logic that more secretion of insulin from tumour may a reason of faster growth of other body cells in pancreatic carcinoma.

Conflict of interest: None

ACKNOWLEDGEMENT

This research work was conducted at the Department of Biochemistry, All India Institute of Medical Sciences, Ansari Nagar, New Delhi (India) and was supported by the Indian Council of Medical Research, Government of India.

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