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### Research Article

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# Low-Density Lipoprotein Receptor Activity in Cultured Human Skin Fibroblasts

## MECHANISM OF INSULIN-INDUCED STIMULATION

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**ABSTRACT** Low-density lipoprotein (LDL) receptor activity, as reflected by LDL degradation, was stimulated by the addition of insulin to cultures of human skin fibroblasts. These changes occurred independently of the glucose concentration of the incubation medium and occurred whether or not LDL receptor activity was suppressed. A comparison of the saturation kinetics of LDL receptor activity in the presence and absence of insulin indicated that insulin produced a 35% increase in  $V_{\max}$  with no difference in "apparent  $K_m$ ". These results suggest that insulin enhances LDL receptor activity by increasing the number of LDL receptors rather than by influencing binding affinity. In confirmation, LDL degradation by receptor negative cells was not enhanced by insulin. Sterol synthesis from [ $^{14}\text{C}$ ]acetate was also stimulated by insulin, but egress of cholesterol and cellular cholesterol content were unaffected by the hormone. The effect of insulin on LDL receptors was not dependent on its known ability to enhance cellular DNA synthesis and proliferation, because insulin stimulated LDL receptor activity in cells kept quiescent by maintenance in plasma-derived serum that was devoid of platelet derived growth factor. Nevertheless, the effect of insulin in enhancing LDL receptor number, coupled with stimulation of endogenous cholesterol synthesis, provides a mechanism whereby the cell could theoretically increase its supply of cholesterol during times of additional need.

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## INTRODUCTION

The receptor-dependent cellular low-density lipoprotein (LDL)<sup>1</sup> pathway appears to be important for the degradation of LDL by a mechanism that also provides the cell with an exogenous source of cholesterol for membrane synthesis (1). The LDL receptor can be finely regulated in response to the requirement of the cell for cholesterol. Thus, when cultured cells are deprived of cholesterol by exposure to a medium containing lipoprotein-deficient serum (LDS), hydroxymethylglutaryl CoA reductase, and cholesterol synthesis are stimulated, and LDL receptor number is increased (2, 3). Conversely, exposure to LDL or cholesterol in the medium results in suppression of receptor activity (2) and in inhibition of cholesterol synthesis.

Insulin also plays an important role in the regulation of many aspects of cellular lipid and lipoprotein metabolism, including an effect on the LDL receptor (4). Fatty acid (5) and cholesterol synthesis (5) are stimulated by the presence of insulin in several cell systems, whereas fatty acid mobilization is inhibited by this hormone (6). Recent observations suggest that insulin also influences cellular lipoprotein metabolism; physiological concentrations result in stimulation of the binding of LDL to its cell surface receptor as well as subsequent internalization and degradation of this lipoprotein (4). Thus, insulin may play a role in both the turnover of circulating LDL and cellular cholesterol metabolism via the LDL pathway.

Insulin potentially could affect several steps in the cellular LDL pathway. The present study was undertaken to elucidate the mechanism by which insulin

<sup>1</sup> *Abbreviations used in this paper:* LDL, low density lipoprotein ( $d = 1.019-1.063$  g/liter); LDS, lipoprotein-deficient serum ( $d > 1.25$  g/liter); PDS, plasma-derived serum; WBS, whole blood serum.

results in stimulation of cellular LDL degradation and to further determine the role of insulin in the regulation of cellular cholesterol metabolism.

## METHODS

**Materials.** Sodium [ $^{125}$ I]iodide (carrier-free in 0.1 M NaOH) and [ $^{14}$ C]Na acetate (57.2 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.). [ $^3$ H]Cholesterol (60.2 mCi/mmol) and [ $^3$ H]thymidine (6.7 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. Insulin (purified single-component pork insulin) was obtained from Eli Lilly & Co., Indianapolis, Ind. Dulbecco-Vogt medium and trypsin were purchased from Grand Island Biological Co. (Grand Island, N. Y.), disposable plastic tissue culture flasks, Petri dishes, and filters from Corning Glassworks, Science Products Div., Corning N. Y. and disposable pipettes from Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif. 25-hydroxycholesterol was obtained from Steraloids Inc., Pawling, N. Y. and cycloheximide from Sigma Chemical Co., St. Louis, Mo.

**Cells.** Cultured human skin fibroblasts were grown from skin biopsies obtained from the anterior thighs of normal volunteers who were neither hyperlipidemic nor diabetic (fasting plasma glucose <115 mg/dl) (7). Receptor-negative cells from patients with homozygous familial hypercholesterolemia (GM 486 and 488) were obtained from The Human Genetic Mutant Cell Repository, Camden, N. J. Cells were grown in monolayer as described (3, 4) and used between the 2nd and 10th trypsinization. Cultured cells were maintained in 250-ml plastic flasks at 37°C in a humidified atmosphere of 95% air, 5% CO<sub>2</sub> in modified Dulbecco-Vogt medium (glucose concentration 300 mg/dl, CaCl<sub>2</sub> = 1.36 mM) containing 10% pooled human serum. The final insulin concentration in the medium was <1 μU/ml as determined by radioimmunoassay (8) of the undiluted serum pool. Confluent monolayers in flasks were dissociated by incubation with 0.05% trypsin for 10 min at 37°C, after which 1 × 10<sup>5</sup> cells were plated into 60-mm diameter plastic Petri dishes containing 4 ml medium. After 7–10 d, by which time the cells were confluent (cell numbers, 1.4–5.4 × 10<sup>5</sup> cell/dish), the medium was removed, and the cells were washed with serum-free medium. The medium was then changed to one containing 10% LDS (prepared from the same plasma pool to which the cells had previously been exposed) to induce LDL receptor activity (2, 3). To test the effect of insulin on lipoprotein cellular interactions, the hormone was added at the same time as the change to LDS-containing medium. Although significant effects on LDL degradation previously have been reported by us to occur at physiological concentrations of insulin (10–100 μU/ml) (4), in the present study, unless otherwise stated, a final concentration of 10,000 μU insulin/ml was used to maximize and also produce consistent insulin effects. In eight paired experiments in which 100 μU/ml insulin was compared with 10,000 μU/ml, stimulation of LDL degradation was higher by a mean of <5% with the larger insulin concentration. In all experiments the test dishes containing insulin were compared with control dishes treated identically in all respects except that no insulin was added. The calcium concentration was optimal for measurement of LDL binding to skin fibroblasts (9), and was the same in the presence and absence of insulin.

**Preparation of  $^{125}$ I-labeled LDL and LDS.** LDL for incubation with cells was isolated from normolipidemic or hypercholesterolemic donors as described (3, 4). LDL ( $d = 1.019$ – $1.063$ ) was isolated by sequential preparative ultracentrifugation using either a Beckman L5-65B or L5-50 ultracentrifuge with the 60 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.). After centrifugation of the LDL once under identical

conditions, the protein content was determined by the Lowry method (10), using bovine serum albumin as standard. The LDL was then iodinated with  $^{125}$ I by the iodine monochloride method as modified for lipoproteins (11) as described (4, 12). After iodination, the  $^{125}$ I-LDL was dialyzed against 0.1 M NaCl, 0.02 M KI at 4°C. Radioactive LDL was used within 1 wk of preparation and was sterilized by passage through a Millipore filter (0.22-μm pore diameter; Millipore Corp., Bedford, Mass.) immediately before use.

LDS was prepared from pooled human serum by ultracentrifugation at  $d = 1.25$  as described (4). After recentrifugation under identical conditions, the  $d > 1.25$ -fraction was extensively dialyzed, initially against 0.15 M NaCl; 1 mM EDTA, and finally against Ringer's solution. The cholesterol content of 10% LDS so prepared ranged between 0.5 and 0.8 μg/ml (determined by gas-liquid chromatography).

**Measurement of degradation of  $^{125}$ I-labeled LDL by fibroblast monolayers.** LDL receptor activity was assayed as the amount of  $^{125}$ I-LDL degraded by the cultured cells. This assay was chosen because it measures net accumulation of breakdown products of LDL in the incubation medium with time, and reflects in an integrated and magnified fashion, events occurring at the level of the LDL receptor (13). Although a small amount of LDL that has been internalized by nonreceptor-mediated processes may also be detected by this assay, this has been shown to be quantitatively insignificant in cells with normal receptors (14). In experiments in which the time required for the degradation assay (usually 24 h) precluded its use, binding of  $^{125}$ I-LDL, rather than LDL degradation, was assayed.

LDL degradation was assayed as follows: Unless otherwise stated, 24 h before the addition of  $^{125}$ I-labeled LDL the medium was changed to one containing 10% LDS with or without added insulin.  $^{125}$ I-LDL was then added to provide a final concentration of 7.5 μg/ml, unless otherwise indicated. Incubations were performed at 37°C in at least duplicate dishes. After 4 or 24 h, a sample of the medium was taken for the determination of degradation of  $^{125}$ I-LDL as originally described (12). After precipitation of proteins by TCA, free iodide in the TCA-soluble fraction was converted to I<sub>2</sub> using hydrogen peroxide. The iodide was then extracted with chloroform (12). Noncell-associated lipoprotein degradation, measured under identical conditions in cell-free dishes, was subtracted from total degradation to give a measure of cellular degradation. The presence of insulin was shown not to influence noncellular degradation. The cell layer was incubated with 0.05% trypsin for 10 min at 37°C and the resuspended cells counted in a hemocytometer; cellular degradation was expressed per 10<sup>6</sup> cells. The precision of replicate analysis of LDL degradation (determined as  $\sqrt{\Sigma d^2/2n}$ , where  $d$  = difference between replicate and  $n$  = number of observations) gave a coefficient of variation of 6.7%. The cell protein content averaged 0.66 mg/10<sup>6</sup> cells.

**Measurement of binding of  $^{125}$ I-labeled LDL.** Binding of  $^{125}$ I-LDL at 37°C was determined as the radioactivity releasable from the intact cell monolayer by gentle trypsinization as described (4, 12).  $^{125}$ I-Labeled LDL (7.5 μg/ml) was added to cells that had been preincubated with LDS-containing medium in the presence or absence of insulin for 24 h. After incubation of  $^{125}$ I-LDL with the cells for 30 min or 4 h at 37°C, the medium was removed and nonspecifically adsorbed radioactivity removed by five washes with 2 ml 0.1% EDTA (12). The cells were then incubated with 0.05% trypsin for 10 min at 37°C to release radioactivity bound to cell-surface receptors. The suspended cells were centrifuged at 1,000 g for 10 min and the radioactivity determined in an aliquot of the supernate. Binding was expressed as the percentage of added radioactivity releasable by trypsinization per 10<sup>6</sup> cells.

When binding was measured at 4°C (Table II), cells were precooled to 4°C, and then incubated with  $^{125}\text{I}$ -LDL (7.5  $\mu\text{g}/\text{ml}$ ) for 2 h. Bound  $^{125}\text{I}$ -LDL was then released from cells by incubating with dextran sulfate (4 mg/ml) for 1 h (15). The cell layer was then dissolved in 0.1 N NaOH for determination of cellular protein content (10). Binding of LDL at 4°C was expressed as nanograms of protein bound per milligram of cellular protein.

**Incorporation of [ $^{14}\text{C}$ ]acetate into sterol.** Cells were grown to confluence, after which the medium was changed to one containing 10% LDS. After the indicated times of exposure to LDS, [ $^{14}\text{C}$ ]acetate ( $5.4 \times 10^6$  cpm/dish) was added for 2 h incubation at 37°C. The cells were washed and harvested by scraping the dishes with a Teflon policeman (Dupont, E. I. de Nemours & Co., Inc., Wilmington, Del.). Extraction of lipids was performed according to the method of Stein et al. (16). After the aqueous and organic phases were split, the protein content was determined on the pellet by the Lowry technique (10). The organic phase was dried under air and saponified with 1 M ethanolic KOH at 80°C for 1 h to hydrolyze cholesterol esters. An aliquot of the lipid extract was then dissolved in hexane and chromatographed on thin-layer plates using hexane/ether/methanol/glacial acetic acid (78:17:3:2) as the solvent system. The sterol spot was scraped into a counting vial for scintillation counting using Aquasol (New England Nuclear). No spot was visible in the position of cholesterol ester. Cellular cholesterol content was measured by gas-liquid chromatography of the cellular lipid extract using stigmaterol as internal standard. After measurement of free cholesterol, a further aliquot was hydrolyzed in ethanolic KOH for determination of total cholesterol.

**Egress of cholesterol from cells.** Confluent cells were pre-labeled with [ $^3\text{H}$ ]cholesterol according to the method of Stein et al. (17). 1 mCi [ $^3\text{H}$ ]cholesterol was dissolved in 0.1 ml ethanol and added to 90 ml medium containing 10% pooled human serum. To allow the labeled cholesterol to equilibrate with that in the cells, confluent fibroblast monolayers were incubated for 48 h at 37°C with 4 ml of medium containing the [ $^3\text{H}$ ]cholesterol. The medium was then removed and the cells were washed six times with serum free medium to remove adsorbed [ $^3\text{H}$ ]cholesterol. 4 ml medium containing LDS with or without insulin was then added to determine the egress of [ $^3\text{H}$ ]cholesterol from the cells. At intervals during the next 24 h, an aliquot of medium was removed and extracted according to the Folch technique. The radioactivity content of the lipid phase was determined. The cell layer was washed and harvested as described above for determination of cellular protein content by the Lowry technique (10). Cholesterol egress also was evaluated by determining the cholesterol content of LDS before and after 24 h incubation with cells.

**Preparation of plasma-derived serum and whole blood serum for the determination of the effect of inhibition of proliferation on the insulin-induced stimulation of LDL degradation.** Plasma-derived serum (PDS) and whole blood serum (WBS) were prepared by the method of Ross et al. (18). Venous blood was drawn from each of five donors into pre-cooled syringes containing 3.8% Na citrate, immediately put on ice and divided so that one-half was processed for PDS and the other half for WBS. WBS was recalcified by adding  $\text{CaCl}_2$  (to give a final concentration of 14 mM), incubated at 37°C for 2 h, and centrifuged at 4°C at 1,000 g for 15 min. The supernate was then recentrifuged at 15,000 g at 4°C for 30 min, decanted, and pooled. The WBS samples were then dialyzed against Ringer's solution for 24 h, heat inactivated for 30 min at 56°C, pooled, and filtered.

To prepare PDS (devoid of the potent mitogenic factors derived from platelets), the other half of the specimen was centrifuged at 1,000 g for 15 min at 4°C. The plasma was

carefully removed to avoid pipetting off any of the leukocyte and platelet layer. The plasma was then respun in prechilled tubes at 15,000 g for 30 min at 4°C to remove any residual cells. The supernate was then clotted by incubating with  $\text{CaCl}_2$  (final concentration 20  $\mu\text{mol}/\text{ml}$ ) for 2 h at 37°C. After recentrifugation at 15,000 g at 4°C for 30 min, the PDS was pooled, dialyzed against Tris-HCl, and then chromatographed on a carboxymethyl cellulose column to remove any residual platelet factor. The PDS so prepared was pretested to ensure adequate removal of mitogenic activity (18).

**Measurements of proliferative response to insulin in PDS and WBS.** Cells were grown to confluence in 10% pooled human serum. The medium was then changed to either 10% WBS or matched PDS for 48 h. This medium was then removed and replaced by 10% LDS, prepared from the serum (either PDS or WBS) to which the cells had previously been exposed. After 24 h exposure to the respective LDS (with or without added insulin), [ $^3\text{H}$ ]thymidine (250  $\mu\text{Ci}/\text{dish}$ ) was added for 4 h incubation at 37°C. The cells were harvested and the incorporation of [ $^3\text{H}$ ]thymidine into TCA-precipitable material performed according to the method of Pollack and Vogel (19).

## RESULTS

Stimulation of LDL degradation by insulin appeared to be independent of the glucose concentration of the medium throughout the range tested. At glucose concentrations ranging from 15 to 600 mg/dl, insulin resulted in equal enhancement of LDL degradation (Fig. 1). In a separate experiment, complete omission of glucose from the medium had no further effect. Cell number was the same in the presence and absence of added insulin. To exclude the possibility that insulin was binding directly to  $^{125}\text{I}$ -LDL, thereby modifying its charge and promoting receptor independent internalization, cells which had not been exposed to insulin previously, were exposed either to regular  $^{125}\text{I}$ -LDL or to  $^{125}\text{I}$ -LDL which had been preincubated with insulin. LDL binding at 4°C (at which temperature internalization does not occur) was measured; no enhanced binding was observed with  $^{125}\text{I}$ -LDL preincubated with insulin. Further confirmation that insulin was enhancing LDL degradation via the LDL receptor and the LDL pathway was obtained by determining LDL degradation in two normal fibroblast strains and in two strains of receptor negative fibroblasts. Whereas in the normal fibroblast controls, degradation of LDL was greater in the presence of insulin (62 and 37%, respectively), the absolute level of LDL degradation by receptor negative cells was extremely low and was not stimulated by insulin (Table I).

In the experiments described above, cells were preincubated with LDS and insulin for 24 h before the addition of  $^{125}\text{I}$ -LDL. Insulin was also present in the medium for the duration of the incubation with labeled lipoprotein. To determine whether shorter periods of exposure to insulin would also influence the LDL receptor, an experiment was performed in which cells were exposed to insulin for brief intervals before the

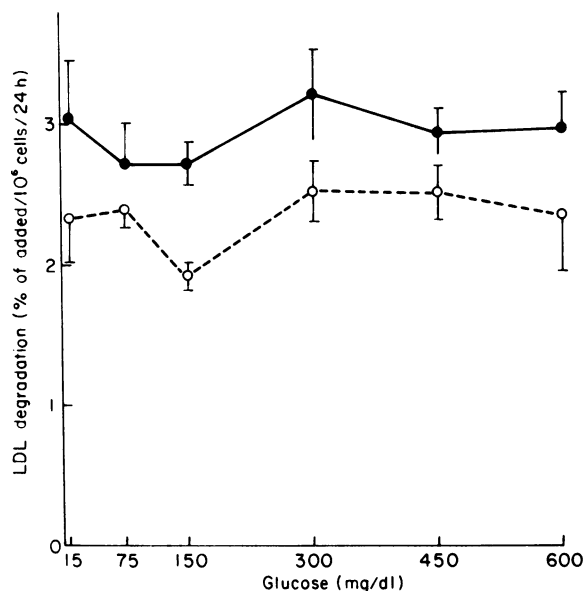


FIGURE 1 Effect of medium glucose concentration on insulin-induced stimulation of LDL degradation. Cells were grown to confluence in medium containing 300 mg/dl glucose. The medium was then changed to one containing the glucose concentration indicated. After 5 d exposure to this glucose concentration, the medium was changed to 10% LDS at the glucose concentration indicated with (●) or without (○) added insulin (10,000  $\mu$ U/ml). 7.5  $\mu$ g/ml <sup>125</sup>I-LDL (166 cpm/ng) was added 24 h later for the determination of LDL degradation over the ensuing 24 h. Values shown are mean  $\pm$ SD of quadruplicate determinations.

addition of <sup>125</sup>I-LDL. To evaluate these shorter time periods, LDL binding was assessed after 30 min exposure of the cells to <sup>125</sup>I-LDL. As has been our previous experience (3, 20), when the medium is changed from one containing pooled human serum to another containing LDS, there appears to be an acute induction of LDL binding (Fig. 2). After reaching a peak at 3 h, binding falls by 6 h, after which it again increases. In the presence of insulin, enhanced binding relative to

the non-insulin controls was observed as early as 2 h and remained present throughout the 24 h of the experiment (Fig. 2).

To determine whether insulin influenced the rate of stimulation of receptor activity, LDL receptors were initially suppressed by exposure to 10% whole pooled human serum. The cells were then washed and exposed to 10% LDS serum in the presence and absence of insulin. <sup>125</sup>I-LDL was added at intervals thereafter for the determination of LDL degradation. The rate of increase of LDL degradation on exposure of cells to LDS was greater in the presence of insulin than when the hormone was absent from the incubation medium (Fig. 3). Thus, insulin appears to enhance the rate of receptor expression during exposure of cells to a cholesterol-deprived medium.

To evaluate whether insulin-induced stimulation of LDL receptor activity was caused by an increased affinity of LDL for its receptor or caused by an increase in the number of available receptors, LDL concentration curves were compared in the presence and absence of insulin using LDL degradation as an assay of receptor activity. Increasing the amount of added <sup>125</sup>I-LDL resulted in saturation of LDL receptor activity both in the presence and absence of insulin (Fig. 4A). At all concentrations of added LDL, receptor activity was greater in the presence of insulin. Linearization techniques (21) were employed to calculate the apparent  $K_m$  and  $V_{max}$  of LDL receptor activity (Fig. 4B). The major effect of insulin was to increase the apparent  $V_{max}$  from 2.91 to 4.44  $\mu$ g/10<sup>6</sup> cells/24 h, while no difference between apparent  $K_m$  was observed in the presence and absence of insulin (9 and 8  $\mu$ g/ml LDL protein, respectively).

To exclude the possibility that the increased receptor activity in the presence of insulin was caused by an effect of the hormone on the rate of degradation of the LDL receptor itself, an experiment was performed in which the rate of loss of receptor activity was measured in the presence and absence of insulin. Receptor

TABLE I  
Effect of Insulin on LDL Degradation in Normal and Receptor Negative Fibroblasts\*

Cell type	Degradation			
	Normal No. 1	Normal No. 2	Receptor negative No. 1	Receptor negative No. 2
	% added LDL/10 <sup>6</sup> cells/24 h			
Insulin added	7.75 $\pm$ 0.20†	10.20 $\pm$ 0.41†	0.16 $\pm$ 0.10§	1.10 $\pm$ 0.15§
No insulin added	4.78 $\pm$ 0.09	7.45 $\pm$ 0.15	0.14 $\pm$ 0.04	1.03 $\pm$ 0.18

Values are mean $\pm$ SD of quadruplicate dishes in which cells were incubated with <sup>125</sup>I-LDL for 24 h.

\* 10,000  $\mu$ U/ml insulin.

†  $P < 0.001$  compared with no insulin added.

§ Not significantly different from no insulin added.

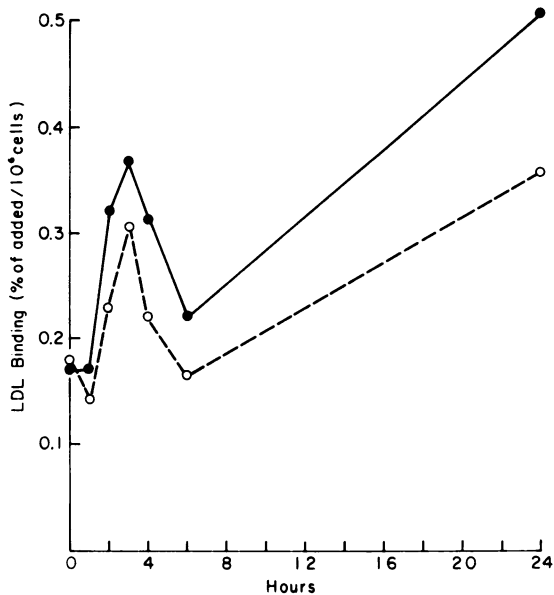


FIGURE 2 Time-course of stimulation of LDL binding by insulin. Confluent cells were washed and the medium changed to 10% LDS with (●) or without (○) insulin (10,000  $\mu$ U/ml). 7.5  $\mu$ g/ml <sup>125</sup>I-LDL (91 cpm/ng) was added at the times indicated for the determination of its binding 30 min later.

activity was first increased to high levels by exposing cells to 10% LDS for 24 h. The cells were then washed and the medium changed to LDS and 0.5 mM cycloheximide either with or without added insulin. <sup>125</sup>I-LDL was added at intervals over the next 50 h for determination of 30 min binding at 4°C. Both in the

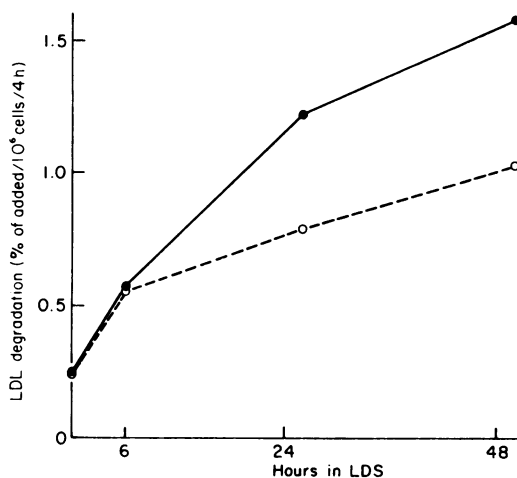


FIGURE 3 Effect of time of exposure to LDS on insulin-induced enhancement of LDL degradation. Cells were grown to confluence in 10% pooled human serum. At time zero the medium was changed to 10% LDS with (●) or without (○) insulin (10,000  $\mu$ U/ml). 7.5  $\mu$ g/ml <sup>125</sup>I-LDL (250 cpm/ng) was added at the times indicated for determination of LDL degradation over the ensuing 4 h.

presence and absence of insulin, LDL binding activity decreased with increasing time of exposure to cycloheximide. Exposure to insulin did not influence the rate of decline of LDL receptor activity, which had half-lives of 79 and 71 h in the presence and absence of insulin, respectively.

A series of experiments was then performed to evaluate whether insulin-induced changes in cellular cholesterol metabolism could possibly account for the changes observed in LDL degradation and LDL receptor activity. The effect of insulin on the egress of cholesterol from cells was studied by prelabeling confluent fibroblasts with [<sup>3</sup>H]cholesterol for 48 h, by which time it was assumed that [<sup>3</sup>H]cholesterol had equilibrated with cellular cholesterol. The cells were then washed and the medium was changed to one containing 10% LDS for the determination of appearance of [<sup>3</sup>H]cholesterol in the medium over the ensuing 24 h. During this time period, the presence of insulin in the medium failed to result in any detectable difference in the egress of [<sup>3</sup>H]cholesterol (Fig. 5). Insulin at both 100 and 10,000  $\mu$ U/ml also did not influence the increase in cholesterol content of LDS after 24 h exposure to cells (Table IIA). The effect of insulin on cholesterol synthesis was also evaluated. To stimulate cholesterol synthesis, cells were grown to confluence in 10% whole pooled human serum and then deprived of a source of exogenous cholesterol supply by a change of the medium to 10% LDS. Cholesterol synthesis was evaluated by pulse labeling with [<sup>14</sup>C]acetate after 4, 24, 48, and 72 h exposure to LDS with or without insulin. Depriving the cells of exogenous cholesterol resulted in a time-related increase in cholesterol synthesis; the presence of insulin resulted in approximately a threefold magnification of these changes by 72 h (Fig. 6). The addition of insulin in medium containing LDS did not increase cell total cholesterol content despite simultaneous stimulation of LDL binding (Table II). Cellular content of unesterified cholesterol showed a slight but statistically insignificant increase in the presence of insulin (Table IIB).

All the experiments described thus far were performed with cells that had been deprived of exogenous cholesterol. To determine whether insulin stimulated LDL degradation under circumstances where cells were not cholesterol depleted, an experiment was performed in which cells were exposed to one of four test media (in the presence and absence of insulin) for 24 h before the addition of <sup>125</sup>I-LDL. Cells exposed for 24 h to 10% LDS acted as cholesterol-depleted controls. To suppress LDL receptor activity, cells were exposed for 24 h to either 10% pooled human serum or 25-hydroxycholesterol (2  $\mu$ g/ml) and cholesterol (20  $\mu$ g/ml) dissolved in 10  $\mu$ l ethanol. A further set of controls had 10  $\mu$ l ethanol together with 10% whole pooled human serum. The presence of 25-hydroxycholesterol and cholesterol

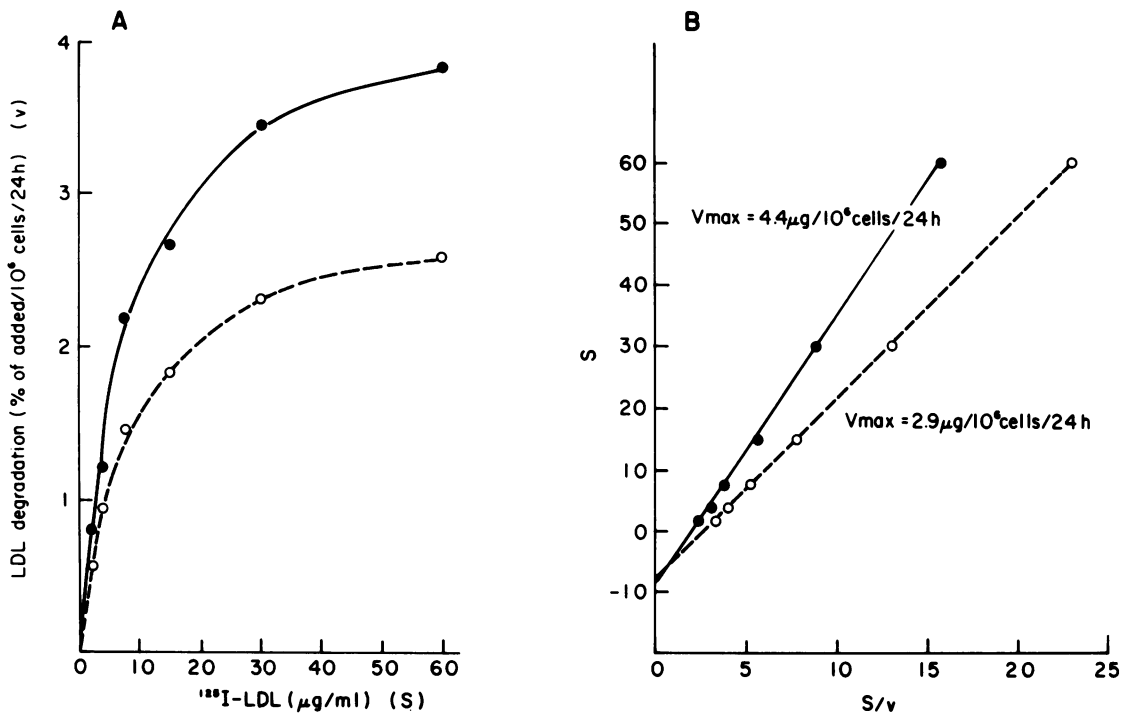


FIGURE 4 Effect of insulin on the kinetics of LDL degradation. Cells were grown to confluence, then preincubated in 10% LDS with or without insulin for 24 h. <sup>125</sup>I-LDL was then added at the concentrations indicated for the determination of LDL degradation over the ensuing 24 h. (A) <sup>125</sup>I-LDL saturation curves in the presence (●) and absence (○) of insulin (10,000 μU/ml). (B) Linearization plot (20) of the data in (A). The slope of the line = V<sub>max</sub>, while the point of intersection with the y axis = -K<sub>m</sub>.

resulted in a reduction of LDL degradation compared to cells exposed to 10% LDS (Table III). However, those cells exposed to insulin still degraded 36% more LDL than cells not exposed to the hormone, compared

with a 38% stimulation by insulin in the control dishes exposed to ethanol alone and 47% stimulation by insulin in cells previously exposed to LDS. Insulin also stimulated LDL degradation in cells exposed to pooled human serum, but the percentage of stimulation was less (14%). Thus insulin at supraphysiological concentrations appears to enhance LDL degradation even in situations where the LDL receptor is partially suppressed.

Because insulin stimulated both LDL receptor activity and endogenous cholesterol synthesis, and because insulin is known to stimulate DNA synthesis and cellular proliferation (22), an experiment was performed to determine whether stimulation of the cell cycle was a prerequisite for the insulin-induced stimulation of LDL receptor activity. To achieve quiescence, confluent cells were transferred to medium containing PDS, which is virtually devoid of the potent platelet derived mitogenic factor (18). Control dishes, exposed to WBS prepared from the same pool of donors, had platelet factor present. After 48 h exposure to PDS or WBS, the medium was changed to LDS previously prepared from the pooled PDS or WBS so as to stimulate LDL receptor activity. [<sup>3</sup>H]Thymidine incorporation into DNA was inhibited in the presence of PDS and insulin (Table IV). By contrast, insulin markedly stimulated the incorporation of [<sup>3</sup>H]thymidine into DNA by cells

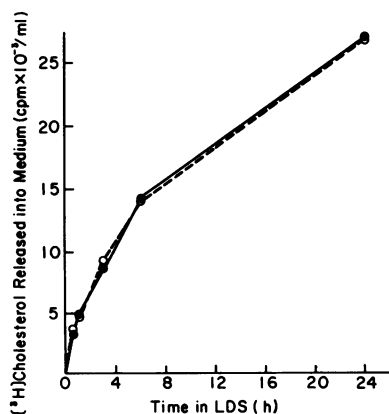


FIGURE 5 Effect of insulin on egress of [<sup>3</sup>H]cholesterol from cultured fibroblasts. Confluent cells were prelabelled by 48 h exposure to [<sup>3</sup>H]cholesterol. The cell layer was then extensively washed and the medium switched to 10% LDS with (●) or without (○) insulin (10,000 μU/ml). Lipid soluble radioactivity was then determined in duplicate aliquots of medium at the times indicated.

TABLE II  
Effect of Insulin on Cellular and Medium Cholesterol Content and LDL Binding

(A)	Medium total cholesterol	
	μg/ml	
No exposure to cells	0.50±0.10	
24 h exposure to cells/0 insulin	0.86±0.02	
24 h exposure to cells/100 μU/ml insulin	0.82±0.06	
24 h exposure to cells/10,000 μU/ml insulin	0.89±0.03	

(B) 24 h exposure to:	Cellular cholesterol	
	Total cholesterol	Unesterified cholesterol
	μg/mg cellular protein	
LDS alone	49.0±7.9	35.1±3.0
LDS + 100 μU/ml insulin	47.2±5.2	37.1±4.7
LDS + 10,000 μU/ml insulin	49.1±6.0	40.4±4.0

(C)	LDL binding at 4°C
	ng/mg cellular protein
LDS alone	121±5
LDS + 100 μU/ml insulin	183±6
LDS + 10,000 μU/ml insulin	190±4

Values shown are mean±SD of quadruplicate determinations.

exposed to platelet factor present in WBS. Cell number under the conditions of the experiment (i.e., confluent cells and LDS) were not influenced by insulin. Despite the marked difference in the DNA synthetic response to the hormone in these two media, insulin nonetheless resulted in stimulation of LDL binding and degradation to approximately the same extent both in the presence and absence of platelet factor (Table IV). This suggests that stimulation of DNA synthesis and cellular proliferation by insulin are not required for the hormone to stimulate LDL receptor activity.

Finally, two experiments were performed to determine whether the effect of insulin in stimulating LDL degradation was dependent on the density of the cells in the dish. First, cells were plated at different densities ( $10^4$ – $10^6$  cells seeded) in like-sized dishes (60-mm diameter). 2 d later the medium was switched to LDS with or without insulin. After a further 24 h,  $^{125}\text{I}$ -LDL was added for determination of its degradation. An inverse relationship between LDL degradation and cell density was observed (Fig. 7A). Insulin did not enhance LDL degradation when the final cell density was  $<10^5$  cells per dish. At higher cell numbers insulin enhanced LDL degradation by 10% at  $1.5 \times 10^5$  cells, 22% at  $5.1 \times 10^5$ , and 30% at  $9.4 \times 10^5$  cells (Fig. 7A). In the second experiment, an equal number of cells (i.e.,  $2 \times 10^5$ /dish) were plated into replicate 35-, 60-, and 90-mm diameter dishes and the same protocol followed

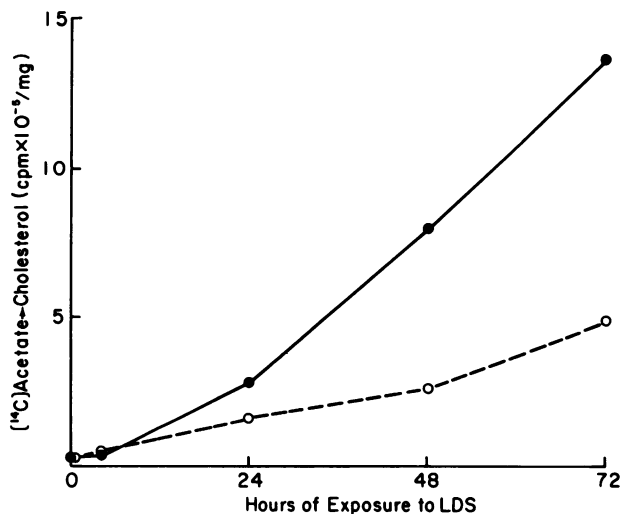


FIGURE 6 Effect of insulin on sterol synthesis by cultured cells. Cells were grown to confluence in 10% pooled human serum. After washing the cell layer, the medium was changed to 10% LDS with (●) or without (○) insulin (10,000 μU/ml). At the times indicated the cells were pulsed with  $^{14}\text{C}$ acetate ( $5.4 \times 10^6$  cpm/dish) for 2 h at 37°C for the determination of acetate incorporation into cellular sterols.

as for the previously described experiment. Mean cell numbers at the end of the experiment were  $1.8 \times 10^5$ ,  $2.2 \times 10^5$ , and  $2.8 \times 10^5$  for the small, medium, and large dishes, respectively, and cells appeared confluent, subconfluent, and sparse in these three dish sizes. Again, in this experiment LDL degradation was greater in the least confluent dishes. Maximal stimulation of LDL degradation was observed in the 35-mm dishes in which the cells were confluent. The least confluent cells (100-mm dishes) showed the least percentage of stimulation of LDL degradation by insulin (Fig. 7B). These findings suggest that the use of subconfluent cultures would not result in a greater magnification of insulin's effect on LDL degradation than that observed with confluent cells.

TABLE III  
Effect of Insulin on LDL Degradation using Incubation Media with Varied Sterol Content\*

	10% LDS	10% PHS	25 OHC + C	Ethanol
	% added $^{125}\text{I}$ -LDL degraded in 24 h/ $10^6$ cells			
No insulin	6.85	1.97	3.98	7.21
Insulin present	10.04	2.24	5.43	9.94
Percentage of stimulation in presence of insulin	47	14	36	38

25 OHC + C, 25-hydroxycholesterol + cholesterol. Values represent the mean of duplicate determinations, the coefficient of variation for which was 6.7%.

\* 10,000 μU/ml insulin.



TABLE IV  
Effect of Insulin on LDL Receptor Activity and DNA Synthesis

	LDL binding (4h)		LDL Degradation		[ <sup>3</sup> H]Thymidine incorporation		Cell number after 48h exposure to LDS	
	PDS	WBS	PDS	WBS	PDS	WBS	PDS	WBS
	% added LDL/10 <sup>6</sup> cells		% added/10 <sup>6</sup> cells/24h		cpm/10 <sup>6</sup> cells/4h		No. × 10 <sup>-6</sup>	
No insulin	0.037	0.045	0.41	0.53	2670	3,652	1.39	1.75
Insulin	0.066	0.078	0.53	0.79	868	15,693	1.43	1.76
% Stimulation by insulin	78.4	73.3	29.3	49.1	-67.5	329.7	2.9	0.6

After growth to confluence in either 10% PDS or WBS, the medium was changed to one containing 10% LDS prepared from the PDS or WBS with or without added insulin (10,000 μU/ml). 24 h later <sup>125</sup>I-LDL was added for determination of its binding at 4h or degradation over 24h, or [<sup>3</sup>H]thymidine was added for measurement of its incorporation into DNA.

## DISCUSSION

Results in this study confirm and extend the finding that insulin enhances the degradation of LDL by cultured human skin fibroblasts (4) and provides support for a possible mechanism. We have previously reported that insulin stimulated LDL degradation and binding in a dose-dependent fashion within the physiological

range of circulating insulin levels (10–100 μU/ml). Furthermore, LDL binding, uptake, and degradation were stimulated concurrently and to a similar extent by insulin (4). Therefore, it is unlikely that the primary effect of insulin on LDL degradation is caused by stimulation of lysosomal enzymes. Were this the case, then expansion of the intracellular regulatory pool of unesterified cholesterol (resulting from hydrolysis of LDL

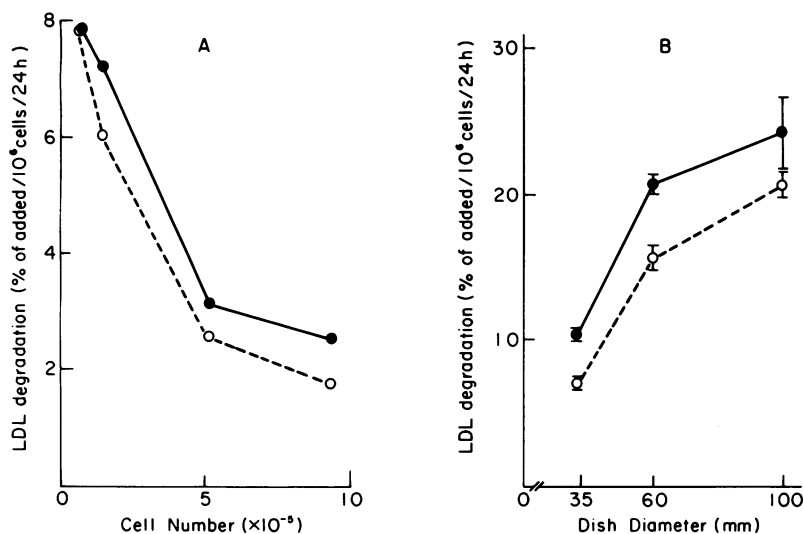


FIGURE 7 Effect of cell density of insulin-mediated stimulation of LDL degradation. (A) Different cell densities were achieved by plating different numbers of cells into like-sized dishes (60-mm diameter). (B) The same number of cells (i.e.,  $2 \times 10^6$ ) were plated into different-sized dishes to achieve different degrees of cell density (● = insulin (10,000 μU/ml); ○ = no added insulin). In both experiments the amount of LDL degraded was inversely related to the cell density in the dish. Also, insulin resulted in greater stimulation of LDL degradation in dishes in which cells were confluent than in those with relatively lower cell numbers. Each data point in A represents the mean of duplicate determination; the specific activity of the LDL used was 173 cpm/ng. In B, each data point is the mean ± SD of quadruplicate values; LDL specific activity was 107 cpm/ng.

cholesterol esters) would be expected to inhibit rather than stimulate the LDL receptor (2, 3). Further, in the absence of the LDL receptor, no insulin-induced enhancement of degradation was observed. Thus, insulin appears to enhance LDL degradation by stimulation of the receptor-dependent LDL pathway. Because LDL degradation was equally stimulated by insulin throughout the glucose concentration range tested and because no further effect was observed in glucose-free medium, this suggests that these changes are independent of an effect of insulin on glucose transport. Although most of these studies were performed at supraphysiological concentrations of insulin (10,000  $\mu\text{U/ml}$ ), previous studies from our laboratory have shown little additional effect on LDL degradation of this concentration when compared with a more physiological concentration (100  $\mu\text{U/ml}$ ) (4). Also, where both 100 and 10,000  $\mu\text{U/ml}$  were used in the same experiment, very similar effects resulted (Table II).

Analysis of LDL concentration curves shows that LDL receptor activity is saturable both in the presence and absence of insulin, but that receptor activity in the presence of insulin is greater at all concentrations of LDL. The major effect of insulin appears to be an increase in apparent  $V_{\text{max}}$  without a change in apparent  $K_m$ . These findings strongly support the notion that insulin increases the number of LDL receptors rather than alters the affinity of the lipoprotein for its cell surface receptor or induces a second LDL receptor of differing affinity. The similar half-lives of receptor activity after cycloheximide treatment suggests that the effects of insulin on the LDL receptor cannot be explained by a change in the rate of degradation of the LDL receptor itself. The stimulation of LDL binding by insulin was rapid, occurring as early as after 2 h of exposure to insulin, suggesting that receptor synthesis is rapidly induced.

Regulation of the LDL receptor *in vitro* is achieved to a large extent by factors which influence the availability of cellular cholesterol (2). Thus, deprivation of exogenous cholesterol supply by exposure of cells to lipoprotein-deficient serum results in an increase in LDL receptor activity (2, 3) presumably by altering the intracellular regulatory pool of unesterified cholesterol (1). Conversely, abundant availability of cholesterol either as LDL (2, 3) or as oxygenated cholesterol derivatives (23) results in down regulation of the LDL receptor. Conditions that deplete this putative regulatory pool of unesterified cholesterol, in addition to stimulating the LDL receptor, also result in stimulation of endogenous cholesterol synthesis (1). It is possible that insulin acts by influencing this cellular cholesterol pool. Previously, insulin has been shown to stimulate cholesterol synthesis by cultured cells by enhancing hydroxymethylglutaryl CoA reductase ac-

tivity in serum-free medium (5). These findings have been extended in the present study during exposure of cells to LDS. The observation that insulin results in stimulation of LDL receptor activity and cholesterol synthesis are compatible with insulin initiating these events by reducing this regulatory microsomal pool of unesterified cholesterol. Theoretically, this could result from stimulation of the egress of cholesterol from cells by insulin. However, no effect of the hormone on egress of [ $^3\text{H}$ ]cholesterol was demonstrable in this system nor was the cholesterol content of the medium higher after changing to LDS containing medium in the presence of insulin (Table II). Since stimulation of LDL degradation also was observed during growth in whole pooled human serum (rich in cholesterol), and during exposure of cells to 25-hydroxycholesterol and cholesterol, it appears that the effect of insulin to enhance the supply of cholesterol via stimulation of the LDL pathway, does not only occur during times of cholesterol deprivation. Thus, a direct effect of insulin on receptor protein synthesis seems the most plausible explanation for the data obtained. However, cholesterol availability may be important in determining the extent to which insulin can stimulate LDL receptor activity. For instance, the extent of insulin-mediated stimulation of LDL degradation was greater in lipoprotein-deficient serum than in matched pooled whole serum (Table III). Despite the lack of effect of insulin on cholesterol egress, and despite simultaneous stimulation of LDL receptor activity (Table II) and enhanced cholesterol synthesis occurring under identical conditions, cells exposed to insulin in LDS did not accumulate significantly more cholesterol than those not exposed to the hormone. Presumably this is partly the result of incubation of the cells in LDS, which is essentially devoid of LDL. Thus, the only LDL available for internalization is the radioactive tracer. Further, any extra cholesterol made available by stimulation of endogenous synthesis would be small when compared against a high background of cell membrane cholesterol and hence undetectable by the methodology used.

Since a well known effect of insulin is the stimulation of cellular growth and proliferation (22), potential enhancement of both exogenous (via the LDL pathway) and endogenous cholesterol supply (via endogenous synthesis) by the hormone could provide the additional cholesterol required for new membrane synthesis. However, under the conditions of these experiments using high cell density and short-term exposure to the hormone, insulin did not stimulate either cell number or cellular protein content (data not shown). Also, neither cell number nor the extent of stimulation of LDL degradation were increased by insulin at lower cell densities. Therefore, to test whether the stimulation of LDL degradation by insulin merely reflected

an increased cellular turnover not detectable by changes in cell number or protein content, an experiment was designed in which the effect of insulin on LDL degradation was compared under conditions of quiescence and active DNA synthesis. Quiescence without serum starvation was achieved by incubation of a confluent cell monolayer with PDS devoid of the potent mitogenic platelet factor (18). Active proliferation was maintained by using matched WBS prepared in such a way as to preserve platelet factor activity. The medium was then changed to LDS from either PDS or WBS to stimulate LDL receptor activity. Despite the marked disparity in DNA synthetic response to insulin between cells exposed to PDS and WBS, LDL degradation was stimulated to a similar degree nonetheless. Cell number was not influenced by insulin, presumably because of the lack of cholesterol in the incubation medium. Thus, neither DNA synthesis nor cellular proliferation appears to be a prerequisite for insulin-induced stimulation of LDL degradation and the findings observed cannot merely be attributed to stimulation of the cell cycle. Furthermore, in the experiments to test the effect of cell density on insulin-mediated stimulation of LDL receptor activity, less stimulation of LDL degradation was observed in sparse cultures than in confluent (and hence density-dependent inhibited cells). The most likely explanation for these observations relates to the inverse relationship between LDL receptor activity and cell density observed in the present study and by others (24–26). It is possible that high basal levels of LDL receptor activity in sparse cultures may represent maximum or near maximum receptor availability. In that case, little further stimulation of LDL receptor activity would be possible. More confluent dishes, with lower basal LDL receptor activity, would then have greater potential for stimulation of receptor number.

Thus, insulin appears to be capable of enhancing the supply of cholesterol to cells by stimulating both endogenous synthesis and also by stimulating LDL transport via the LDL receptor. The increased cholesterol potentially made available as a result of these two processes could, under appropriate conditions, presumably be used during times of cholesterol deprivation or during times of active cellular growth and proliferation. Enhanced lysosomal degradation of LDL usually accompanies stimulation of LDL binding and stimulation of cholesterol synthesis (1). Therefore insulin may have a dual role in cellular lipid and lipoprotein metabolism. On the one hand, insulin theoretically could increase the availability of cellular cholesterol, while on the other it stimulates the physiological pathway of LDL degradation *in vitro*. Since LDL degradation *in vivo* is believed to be mediated largely via this receptor-mediated pathway,

it is conceivable that changes in circulating insulin levels could also modulate LDL degradation *in vivo* if fibroblasts are a valid model for peripheral LDL degradation. This possibility remains to be tested.

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