Low density lipoprotein receptors in rat adipose cells: subcellular localization and regulation by insulin

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Abstract The distribution of LDL receptors within subcellular compartments of isolated rat adipose cells and the effects of insulin on their expression have been assessed. By immunoblotting with specific anti-rat LDL receptor antibodies, LDL receptors were 2.3- and 4.5-fold enriched in endoplasmic reticulum-rich high-density microsomes (HDM) and Golgi complex-rich low-density microsomes (LDM), respectively, compared to plasma membranes (PM). This distribution was similar in cultured cells in which total receptors were increased 2.5-fold compared to freshly isolated cells. After correction for enzyme recoveries, LDL receptors were distributed ~4% in HDM, ~73% in LDM, and ~23% in PM. Insulin decreased total LDL receptors in adipose cells ~44%, with a 48% and 49% decrease in HDM and LDM, respectively, without any changes in PM. In contrast, insulin caused an increase of glucose transporters in PM while also decreasing glucose transporters in LDM. When adipose cells were depleted of potassium to inhibit receptor-mediated endocytosis, insulin again caused a decrease of LDL receptors in LDM but now increased LDL receptors in PM. Insulin increased the rate of LDL receptor synthesis ~24%, but decreased their half life ~40%. Thus, in isolated adipose cells the majority of LDL receptors appear to be located in an intracellular compartment that co-sediments with the Golgi complex rather than located in the PM. The LDL receptors localized in intracellular compartments seem to be functionally regulated as insulin acutely diminishes the number of receptors by apparently accelerating their rate of degradation through, as yet, incompletely determined mechanisms.—**Kraemer, F. B., S. A. Sather, B. Park, C. Sztalryd, V. Natu, K. May, H. Nishimura, I. Simpson, A. D. Cooper, and S. W. Cushman.** Low density lipoprotein receptors in rat adipose cells: subcellular localization and regulation by insulin. *J. Lipid Res.* 1994. **35:** 1760–1772.

Supplementary key words subfractions • synthesis • half-life • rat

The low density lipoprotein (LDL) receptor is a glycoprotein that mediates the uptake of apolipoprotein B- and E-containing lipoproteins. The LDL receptor has been extensively characterized and shown to be present in most mammalian tissues by ligand binding studies (1), immunological studies (2, 3), or by the presence of LDL receptor mRNA (4). A limited number of studies have examined the LDL receptor in adipose cells and have yielded somewhat conflicting results. Specific LDL binding has been observed in membranes prepared from bovine adipose tissue (1); however, LDL binding in human adipose cells and adipocyte membranes was found to differ from classical LDL receptors by displaying calcium independence, resistance to proteolytic destruction, and a "relaxed lipoprotein specificity" as HDL and methylated LDL effectively competed with native LDL, suggesting the presence of a different class of receptor (5). In contrast to these results, examination of a mouse adipocyte cell line and primary mouse adipose cells revealed LDL receptors with lipoprotein specificity similar to those in fibroblasts (6). The LDL receptor is localized on the surface of most cells in coated pits where, after binding, the lipoprotein-LDL receptor complex is rapidly internalized by adsorptive endocytosis (7). The internalized lipoprotein-LDL receptor complex rapidly dissociates within endosomes, allowing the ligand-free LDL receptor to recycle back to the cell surface. The number of LDL receptors ex-

Abbreviations: LDL, low density lipoprotein; IGF II, insulin-like growth factor II; LRP, low density lipoprotein receptor-related protein/α_2-macroglobulin receptor; KRBH, Krebs Ringer-bicarbonate HEPES buffer; TES, Tris-EDTA-sucrose buffer; HDM, high density microsomes; LDM, low density microsomes; PM, plasma membranes; CHAPS, cholamidopropyl-dimethylammonio-1-propane-sulfonate.

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pressed by a cell is regulated primarily by cellular sterol content with sterols inhibiting the transcription of LDL receptor mRNA through the interaction with sterol regulatory elements in the LDL receptor gene (8, 9). In addition to sterols, LDL receptor expression is regulated by growth factors and hormones, such as platelet-derived growth factor (10) and insulin (11).

Most studies with fibroblasts and hepatocytes have suggested that up to 90% of LDL receptors are localized to the cell surface and very few reside inside the cell (12). Recently, however, two lines of evidence have suggested that this might not be the case in all instances. First, derivatization of surface receptors in fibroblasts or leukemia cells with sulfosuccinidyl dithiopropionyl agents results in ~15–50% of cellular LDL receptors remaining unlabeled, presumably due to their being located intracellularly (13, 14). Second, immunofluorescence and immunoelectron microscopy of LDL receptors in tissues of transgenic mice overexpressing the human LDL receptor suggest that the distribution of the LDL receptor to specific areas of the cell surface, as well as to intracellular vesicles, varies among tissues (15), although it is possible that the distribution of receptors in transgenic animals is due to the abnormally high level of receptor expression. The presence of a substantial proportion of "cell surface" receptors within intracellular compartments of the cell has been observed for several membrane receptors and transport proteins such as asialoglycoprotein receptors (16), transferrin receptors (17), insulin-like growth factor II (IGF II) receptors (18), α_2-macroglobulin receptors (19), and glucose transporters (20). It is of particular interest that the α_2-macroglobulin receptor has also been localized primarily to an intracellular compartment as this receptor is identical to the low density lipoprotein receptor-related protein (LRP) (21), a protein previously considered by most investigators to be a cell surface receptor. The occurrence of an intracellular pool of membrane receptors or transport proteins has significance because it provides an important mechanism for the rapid regulation of surface receptors and transport proteins through bi-directional movement between the plasma membrane and the intracellular compartment. In this regard, phorbol esters cause a rapid redistribution of asialoglycoprotein and transferrin receptors from the cell surface to the cell interior (22), while insulin stimulates the translocation of transferrin receptors (23), IGF II receptors (24), and α_2-macroglobulin receptors (25), as well as glucose transporters (26) from an intracellular pool to the plasma membrane. The present studies were undertaken to determine whether in rat adipose cells LDL receptors are localized predominantly to the plasma membrane or are present in a significant amount in an intracellular compartment. In addition, as insulin has been reported to increase LDL receptor expression in cultured fibroblasts (11), hepatocytes (27), and mononuclear cells (28), the effects of insulin on LDL receptor expression in a classical insulin-responsive tissue (rat adipose cells) were examined in light of the possibility that insulin might cause the rapid redistribution of an intracellular pool of LDL receptors to the cell surface.

MATERIALS AND METHODS

Adipose cells isolation and membrane preparation

Adipose cells were isolated by collagenase digestion under sterile technique from 180–240 g male Sprague-Dawley rats (Bantin Kingman Co, Fremont, CA) as previously described (26, 29). Collagenase digestion was carried out in Krebs-Ringer-bicarbonate-HEPES (KRBH) buffer (pH 7.4) containing 5% bovine serum albumin (BSA) and 200 nM adenosine. For cell incubations exceeding 4 h, the KRBH buffer was rough filtered through Whatman grade I paper and then through a 0.45-micron filter prior to cell addition. In order to examine the effects of potassium depletion, cells were incubated in KRBH as above, but with 0 mM KCl for 1 h prior to adding insulin. For some long-term incubations, cells were cultured in Eagle's medium (pH 7.4) modified with Earle's salts with glutamine (2 mM), 25 mM $NaHCO_3$, 50 μg/ml gentamycin, 1.25 mM HEPES, and 5% (w/v) BSA. The culture dishes were shaken gently over the course of 0.25–16 h incubations and kept at 37°C under 95% air/5% CO_2. After incubations, the cells were homogenized at 18°C in TES buffer consisting of 20 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 255 mM sucrose, and 100 μM leupeptin. A 1-ml aliquot was taken for protein and enzyme determinations. The cell homogenates were then fractionated by differential ultracentrifugation to yield plasma membranes (PM), high-density microsomes (HDM), and low-density microsomes (LDM), as described previously (26, 30). Intact epididymal fat pads were washed in phosphate-buffered saline, homogenized, and membranes were subfractionated as described for isolated cells without exposure to collagenase.

Immunoblotting and immunoprecipitation

Samples of whole homogenates or membrane proteins were solubilized in CHAPS or Triton X-100 detergent, and electrophoresed under nonreducing conditions on 6% polyacrylamide gels containing 0.1% SDS after the addition of 0.5% SDS and 13% glycerol to the samples, as described previously (31). After electrophoresis and transfer to nitrocellulose paper, the filters were incubated with rabbit polyclonal anti-rat LDL receptor antibodies, prepared as described previously (32), at an approximate final IgG concentration of 10 μg/ml for 2 h at room temperature followed by 350 ng/ml of goat anti-rabbit IgG that had been radiolabeled with ^{125}I by the chloramine T method of Greenwood, Hunter, and Glover (33). The nitrocellulose filters were washed and air-dried overnight

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prior to autoradiography on Kodak XAR film for 5–120 h at −80°C. The relative amounts of immuno-detectable LDL receptor contained in each lane were determined either by excising the corresponding band from the nitrocellulose filter and measuring radioactivity in a γ scintillation counter or by scanning with an LKB Ultrascan XL enhanced laser densitometer and Gelscan XL software (Pharmacia LKB Biotechnology, Piscataway, NJ) on a NEC computer. In some cases LDL receptors were quantitated by ELISA using polyclonal rabbit anti-LDL receptor antibodies as described previously (34).

Metabolic labeling

In order to label cells metabolically, isolated adipose cells were pulse-labeled by incubating for various times in 5 ml of Dulbecco's modified essential media deficient in methionine containing 3% BSA, and 100 μCi/ml of [^{35}S]methionine (Amersham Life Science Products, Arlington Heights, IL) in the presence or absence of insulin and then chased by washing once with DMEM–3% BSA supplemented with 3 mM cold methionine and then incubating in fresh media in the presence or absence of insulin for various times (31, 35). The incubations were performed at 37°C under an atmosphere of 95% air/5% CO_2 with shaking at 60 cycles/min. At the end of the incubations, aliquots of 1×10^6 cells were rapidly separated from the medium by centrifugation in a microfuge through 0.5 ml of silicone oil. Packed cells were collected and placed into 0.5 ml of ice-cold lysis buffer (0.15 M NaCl, 3% Triton X-100, 0.1% lauryl sarcosyl, 1 mM PMSF, 1 unit/ml leupeptin, and 0.2 mg/ml aprotinin). Samples were vortexed, sonicated briefly (3 sec), shaken vigorously for 1 h at 4°C, and then centrifuged at 10,000 *g* for 15 min. The infranatant below the fat and oil cake was used for immunoprecipitation and protein determination. An aliquot of 0.4 ml was precleared with pansorbin and then incubated with rabbit polyclonal anti-rat LDL receptor antibodies (~0.5 μg/ml) at 4°C for 12–14 h. The immune complex was isolated by adding pansorbin (100 μl/tube) for 60 min at 4°C and then centrifuging at 10,000 *g* for 15 min. The pellet was washed twice with a buffer containing 0.02 M HEPES, 0.15 M NaCl, 0.002 M $CaCl_2$, 0.002 M $MgCl_2$, 0.002 M cold methionine, 1 mM PMSF, 1 unit/ml leupeptin, 0.2 mg/ml aprotinin, 1% Triton X-100, 0.5% deoxycholic acid, and 0.1% SDS. The pellet was resuspended in 0.063 M Tris-HCl (pH 6.8) containing 8 M urea, 1% β-mercaptoethanol, 1% SDS, and 13% glycerol, boiled for 5 min and electrophoresed on 10% polyacrylamide gels containing 0.1% SDS. After drying, the gels were exposed to Kodak XAR film at −80°C for 1 h to 3 d for autoradiography.

Other methods

Protein content was determined by the Bio-Rad Protein Assay. Activity of 5'-nucleotidase, a PM marker, was assayed as described by Avruch and Hoelzl-Wallach (36) in the presence of 0.05% Triton X-100 and 5 mM 2',3'-AMP. Rotenone-insensitive NADH-cytochrome C reductase activity, a marker of endoplasmic reticulum (HDM), was assayed using the method of Dallner, Siekevitz, and Palade (37). UDP-galactose:N-acetylglucosamine galactosyltransferase activity, a Golgi marker (LDM), was determined by the method of Fleischer (38) using a 2-(N-morpholino)ethanesulfonic acid buffer, pH 6.5. LDL receptor distribution among the subcellular membrane fractions was corrected on the basis of cross contamination of each of the subcellular fractions. Assuming that each marker enzyme was unique to its respective subcellular fraction, the specific activities of each marker enzyme within each subfraction and the protein recoveries of each subfraction were used to simultaneously solve differential equations that provided the contribution of each "pure" subfraction. The calculations were performed on a program using Microsoft Excel (Microsoft Corp., Redmond, WA) for the Macintosh. (The cross contamination program is available to interested individuals by contacting the corresponding author.) Glucose transporters were measured by the specific binding of [^{3}H]cytochalasin B to membrane fractions in the absence or presence of D-glucose as described previously (39). Statistical analyses were performed using StatView II (Abacus Concepts, Berkeley, CA) on a Macintosh computer.

RESULTS

Because lipoprotein binding assays can be difficult to interpret, particularly with tissues such as adipose cells that contain large quantities of lipids that can interfere with binding or separation of unbound lipoproteins, we used a specific polyclonal antibody raised against ethinyl estradiol-treated rat hepatic LDL receptors to quantify LDL receptors in adipose cells. The specificity and characterization of this antibody has been described previously (32, 34) and the immunoblot displayed in **Figure 1** demonstrates that the protein in rat adipose cells recognized by these anti-LDL receptor antibodies is similar in size (~135 kD) to the LDL receptor from the liver of ethinyl estradiol-treated rats. However, an LDL receptor-like protein, which has been termed the VLDL receptor and that binds VLDL, β-VLDL, and IDL, but not LDL, with high affinity, has recently been cloned from rabbits (40). As the VLDL receptor cDNA encodes a protein of similar size to the LDL receptor, shares ~50% homology with the LDL receptor, and VLDL receptor mRNA is predominantly found in muscle (heart) and adipose tissues, with barely detectable amounts in liver, we sought to explore further the specificity of our anti-rat LDL receptor antibodies. To examine whether our anti-rat LDL receptor antibodies recognized the VLDL receptor,

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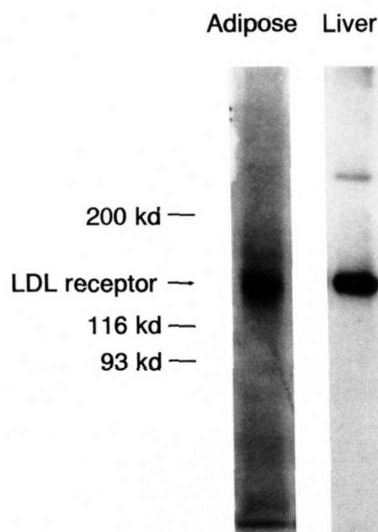

Fig. 1. Immunoblot of LDL receptors in rat adipose tissue and in livers from ethinyl estradiol-treated rats. Detergent extracts of epididymal fat (193 μg) from a control rat and liver (5 μg) from a rat treated with ethinyl estradiol (10 mg/kg) for 3 days were separated on SDS-PAGE, transferred to nitrocellulose, and probed with rabbit polyclonal anti-rat LDL receptor antibodies and ^{125}I-labeled goat anti-rat IgG as described in Materials and Methods. The autoradiograph was developed after 5 h exposure at −80°C.

extracts from hearts of control mice and mice in which functional LDL receptors had been removed by homologous recombination (41) were immunoblotted with anti-LDL receptor antibodies (**Fig. 2**). As reported previously (31), LDL receptors in control mouse liver were ~5,000 kD smaller than the LDL receptor in control rat liver. In addition, immunoreactive LDL receptors were seen in control mouse hearts, as described previously (1); however, no immunoreactive proteins were observed in hearts from LDL receptor knockout mice. Thus, our polyclonal antibody raised against ethinyl estradiol-treated rat hepatic LDL receptors appears to be specific for LDL receptors and does not recognize the VLDL receptor in mice.

Figure 3 illustrates the subcellular distribution of LDL receptors among high-density microsomes (HDM) enriched in endoplasmic reticulum, low-density microsomes (LDM) enriched in Golgi complex, and plasma membranes (PM). Using anti-LDL receptor antibodies, a relative enrichment of immunoreactive LDL receptors in the HDM and LDM was observed (Fig. 3A). The results from nine separate experiments of excising the LDL receptor band from the nitrocellulose filter and measuring the radioactivity showed that the HDM was 2.3-fold enriched in LDL receptors compared to PM and that the LDM was 4.5-fold enriched in LDL receptors compared to PM when compared on the basis of equal amounts of membrane protein from each of the subfractions, suggesting the presence of a large pool of intracellular receptors (Fig. 3B). The specific activities and the activity ratios of the marker enzymes within each subfraction are given in **Table 1.** It is apparent that the subfractionation procedure was successful in enriching each subfraction with its marker enzyme; however, considerable cross contamination remained (see references 26 and 30 for a detailed description and discussion of the isolation and recovery of the subfractions). Total recovery for each of the enzyme markers averaged 124 ± 19% for 5'-nucleotidase, 41 ± 6% for galactosyltransferase, and 31 ± 5% for cytochrome-C reductase. When the subcellular distribution of LDL receptors was recalculated using enzyme and protein recoveries of each of the subfractions and with the assumptions that each of the marker enzymes is found exclusively within a particular subfraction, the HDM was calculated to contain 3.8%, the LDM 72.6%, and the PM 23.7% of the total LDL receptors in adipose cells (Fig. 3C). Thus, the vast majority of LDL receptors in rat adipose cells appear to be located within an intracellular compartment that co-sediments with the Golgi complex.

The low abundance of LDL receptor in the plasma membrane fraction raised the possibility that the col-

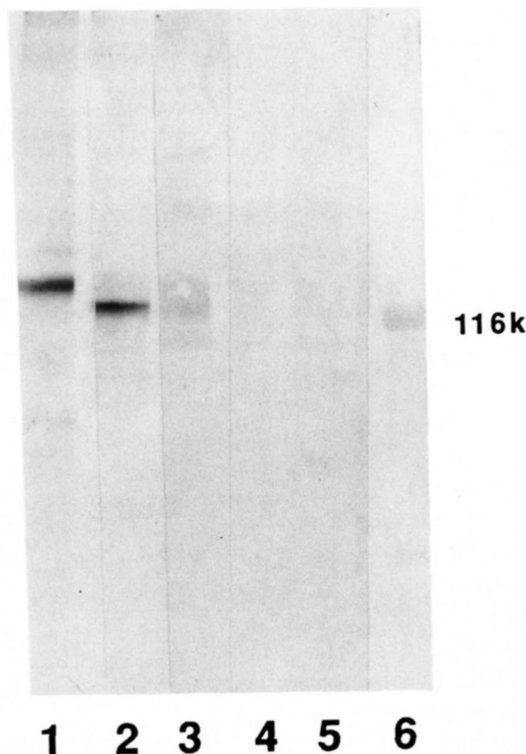

Fig. 2. Immunoblot of LDL receptors in hearts from control and LDL receptor knockout mice. Detergent extracts (100 μg protein) of liver from a control rat (lane 1), from a control mouse (lane 2), of heart (lane 3) from a control mouse and heart (lanes 4, 5) from two LDL receptor knockout mice were separated on SDS-PAGE, transferred to nitrocellulose, and probed with rabbit polyclonal anti-rat LDL receptor antibodies and visualized with horseradish peroxidase. Lane 6 contained a molecular weight marker.

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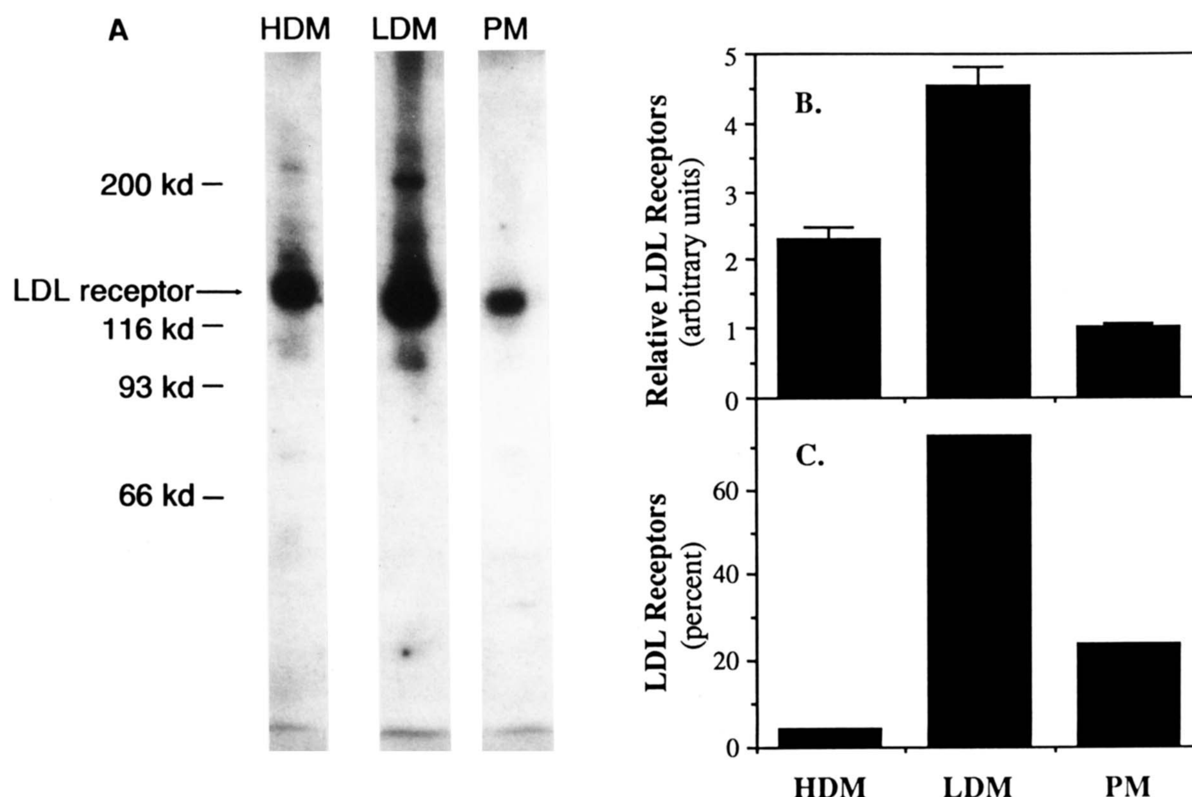

Fig. 3. Distribution of LDL receptors in various subcellular fractions of isolated rat adipose cells. Panel A: Autoradiograph of an immunoblot of LDL receptors in high-density microsomes (HDM), low-density microsomes (LDM), and plasma membranes (PM) prepared by sucrose gradient centrifugation from adipose cells isolated from rat epididymal fat pads. Membrane fractions (35 μg each) were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with rabbit polyclonal anti-rat LDL receptor antibodies and ^{125}I-labeled goat anti-rat IgG as described in Materials and Methods. The autoradiograph was developed after 48 h exposure at $-80°$C. Panel B: Relative distribution of LDL receptors in HDM, LDM, and PM fractions. The LDL receptor band was excised from the nitrocellulose filters of nine separate experiments and the radioactivity was measured; the results are expressed as mean $\pm$ SEM. The lowest amount of LDL receptor was arbitrarily set as 1. Panel C: The percentage of total LDL receptors contained in HDM, LDM, and PM after correction for enzyme recoveries.

lagenase treatment used to isolate the adipose cells might have destroyed the receptors in the plasma membrane. To explore this possibility, adipose cells were maintained in culture without added lipid or lipoproteins, thus allowing for regeneration of plasma membrane receptors. **Figure 4A** demonstrates a 2.5-fold increase in total cellular LDL receptor content over a 16 h incubation as assessed by immunoblotting the cellular homogenates. When cells that had been cultured in lipid-free media for 16 h were subfractionated, the distribution of LDL receptors was quite

TABLE 1. Specific activities and activity ratios of marker enzymes in various subcellular fractions of isolated rat adipose cells

Fraction	5'-Nucleotidase		Galactosyltransferase		Cytochrome-C Reductase	
	SA	Ratio	SA	Ratio	SA	Ratio
	μmol/mg/h	*%*	*nmol/mg/h*	*%*	*μmol/mg/h*	*%*
Hom	0.1 ± 0.0		6.5 ± 2.8		0.5 ± 0.1	
PM	1.2 ± 0.3	100	18.4 ± 8.1	41 ± 10	0.6 ± 0.2	21 ± 6
HDM	0.2 ± 0.1	21 ± 6	15.9 ± 4.5	43 ± 4	3.8 ± 1.0	100
LDM	0.4 ± 0.1	40 ± 13	34.8 ± 9.6	100	1.7 ± 0.1	55 ± 9

Membrane fractions were prepared by sucrose gradient centrifugation of adipose cells isolated from rat epididymal fat pads into whole homogenate (Hom), plasma membranes (PM), high-density microsomes (HDM), and low-density microsomes (LDM). Each fraction was then assayed for enzyme activities of 5'-nucleotidase, galactosyltransferase (UDP-galactose:N-acetylglucosamine galactosyltransferase), and cytochrome-C reductase (rotenone-insensitive NADH-cytochrome C reductase). Results are the mean ± SEM of eight experiments. SA, specific activity; ratio, activity ratio. The activity ratio was calculated by setting the specific activity of a marker enzyme in its representative fraction to 100% and expressing the other fractions relative to that value.

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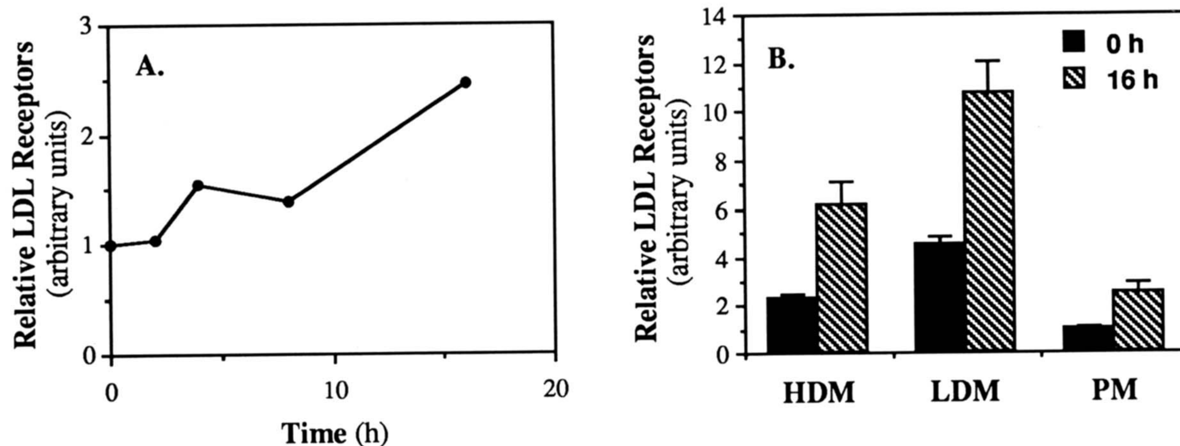

Fig. 4. Time course (A) and relative distribution (B) of LDL receptors in cultured adipose cells. Panel A: Time course of total LDL receptor expression in adipose cells cultured in lipid-free media. Adipose cells isolated from rat epididymal fat were cultured for the indicated times in Eagle's medium modified with Earle's salts with 2 mM glutamine, 25 mM $NaHCO_3$, 50 μg/ml gentamycin, 1.25 mM HEPES, and 5% BSA under an atmosphere of 95% air/5% CO_2 with shaking at 60 cycles/min. At the indicated times cells were removed and detergent extracts of the homogenates were immunoblotted with anti-LDL receptor antibodies as described in Materials and Methods. The amount of LDL receptors detected in freshly isolated cells was arbitrarily set at 1. Panel B: Relative distribution of LDL receptors in various subcellular fractions of isolated adipose cells after 16 h incubation in lipid free media. After cells had been cultured for 16 h in lipid-free media, high-density microsomal (HDM), low-density microsomal (LDM), and plasma membrane (PM) fractions were prepared and incubated with anti-LDL receptor antibodies as in Fig. 3. The LDL receptor band was excised from the nitrocellulose filters of six separate experiments and the radioactivity was measured; the results are expressed as mean ± SEM. The lowest amount of LDL receptor found at 0 h was arbitrarily set as 1.

similar to that observed in freshly isolated cells (Fig. 4B); the HDM was enriched 2.5-fold and the LDM was enriched 4.3-fold compared to the PM when analyzed on the basis of an equal amount of membrane protein. Therefore, the vast majority of LDL receptors was still found in the LDM fraction even though the total number of LDL receptors was substantially greater.

To test further for a possible artifact due to the use of collagenase in isolating the cells, the distribution of LDL receptors in subcellular fractions from freshly isolated adipose cells was compared with subfractions prepared from intact fat pads that had never been exposed to collagenase. The subcellular distribution of LDL receptors was quite similar between the preparations (**Fig. 5**), with the HDM and LDM in the fat pad enriched 4.9-fold and 4-fold in LDL receptors, respectively, compared to PM. Because the intact fat pad is composed of approximately 50% adipose cells, there were some differences in the recovery of enzyme markers; however, even with the limitations of the methods, these data further support the conclusion that most of the LDL receptors in adipose cells are located intracellularly.

Because insulin is known to exert an effect on the translocation of glucose transporters (26) and some cell surface receptors (23–25) from an intracellular pool to the plasma membrane and insulin has been reported to increase LDL receptor activity in other cells (11, 27, 28), the effect of acute exposure of insulin on the level of LDL receptor expression and its distribution in adipose cells was examined. When isolated adipose cells were exposed to insulin for 30 min (a time frame that would enable translocation to be observed), subfractionated, and immunoblotted for LDL receptors, total LDL receptors in cell homogenates decreased by 44 ± 9% ($P < 0.02$). This is a contrast to the increase in LDL receptor levels previously reported to occur in cultured fibroblasts (11) or hepatocytes (27) after exposure to insulin. As shown in **Figure 6,** this insulin-induced decrease in LDL receptors was reflected by a decrease in the abundance of receptors within the HDM (47 ± 9% decrease, $P < 0.001$) and LDM (48 ± 10% decrease, $P < 0.001$) without any consistent changes in

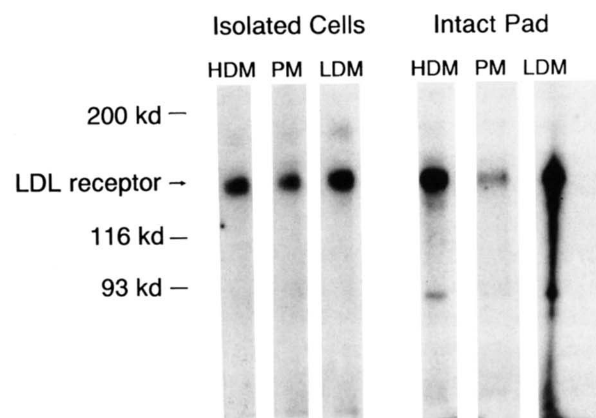

Fig. 5. Immunoblot of LDL receptors in various subcellular fractions prepared from isolated adipose cells and from intact epididymal fat pads. High-density microsomes (HDM), low-density microsomes (LDM), and plasma membranes (PM) were prepared, and fractions (50 μg each) were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with anti-LDL receptor antibodies as in Fig. 3. The autoradiograph was developed after 24 h exposure at −80°C.

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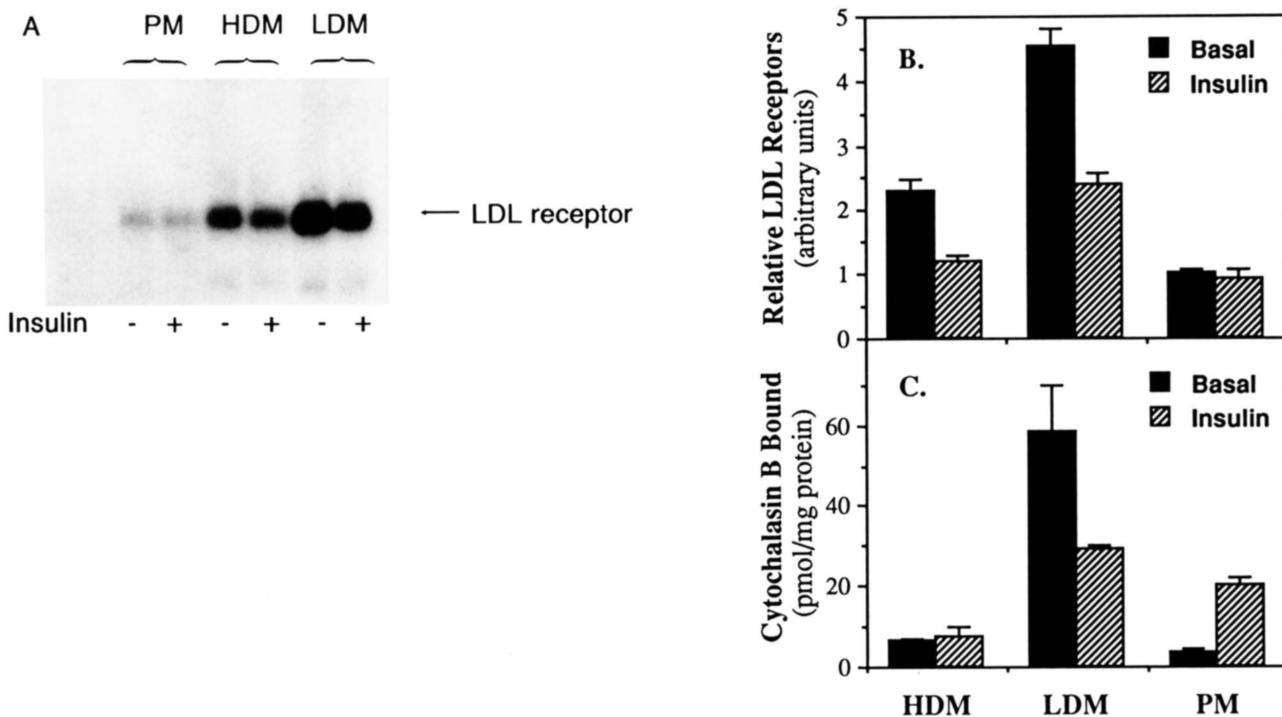

Fig. 6. Effects of insulin on LDL receptor distribution in various subcellular fractions of isolated rat adipose cells. Panel A: Immunoblot of LDL receptors in HDM, LDM, and PM prepared from isolated adipose cells incubated in the presence or absence (basal) of insulin (500 μU/ml) for 30 min. High-density microsomes (HDM), low-density microsomes (LDM), and plasma membranes (PM) were prepared, and fractions (150 μg each) were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with anti-LDL receptor antibodies as in Fig. 3. The autoradiograph was developed after 5.5 h exposure at $-80°C$. Panel B: Relative distribution of LDL receptors in HDM, LDM, and PM fractions from basal and insulin-treated adipose cells. The LDL receptor band was excised from the nitrocellulose filters of nine separate experiments and the radioactivity was measured; the results are expressed as mean $\pm$ SEM. The lowest amount of LDL receptor in PM of basal cells was arbitrarily set as 1. Panel C: Effects of insulin on the distribution of glucose transporters in HDM, LDM, and PM from isolated adipose cells. Glucose transporters were measured by the specific binding of [^{3}H]cytochalasin B binding to membrane fractions in the absence or presence of D-glucose as described in Materials and Methods.

the abundance of LDL receptors in the PM (9 $\pm$ 15% decrease, P = NS). The LDL receptors that were lost following insulin treatment could not be detected within the fat cake or other fractions (nuclei/debris) which were usually discarded (data not shown). In contrast to the effects on LDL receptors, insulin caused a marked increase in the translocation of glucose transporters from the LDM fraction to the plasma membrane (Fig. 6C) without altering the total number of glucose transporters. Thus, insulin appears to cause an overall decrease in LDL receptors in adipose cells without changing the number of LDL receptors in the plasma membrane, while concomitantly increasing the number of glucose transporters in the plasma membrane through translocation. The time course for the insulin-induced decrease in LDL receptors is shown in **Figure 7.** LDL receptors were decreased within 2 min of exposure of cells to insulin, with a maximal effect seen by 10 min. In addition, the insulin-induced decrease in LDL receptors was observed whether adipose cells were exposed to insulin for up to 16 h, or whether isolated adipose cells that were first cultured for 16 h in lipid-free media prior to exposure to insulin were used (data not shown).

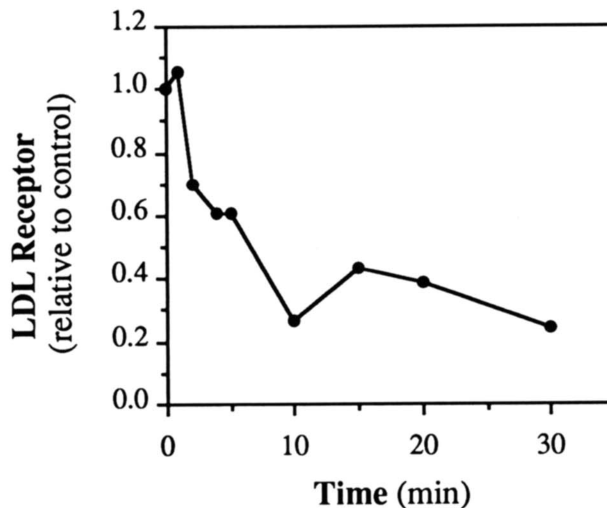

Fig. 7. Time course of insulin action on LDL receptors in isolated rat adipose cells. Isolated adipose cells were exposed to insulin (500 μU/ml) for the indicated times. Total membranes were then prepared, and fractions (100 μg each) were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-LDL receptor antibodies as described in Materials and Methods. The amount of LDL receptors is plotted relative to that detected in control cells incubated in parallel in the absence of insulin.

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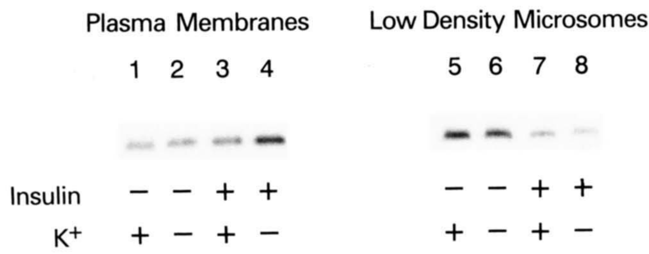

Fig. 8. Effects of potassium depletion and insulin on LDL receptor distribution in subcellular fractions in isolated adipose cells. Isolated adipose cells were maintained either in potassium replete (4 mM KCl, K^+ +) or potassium deplete (0 mM KCl, K^+ −) KRBH for 1 h prior to an additional 30 min incubation in the presence or absence (basal) of insulin. Plasma membranes (PM) and low-density microsomes (LDM) were then prepared, and fractions (150 μg each) were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with anti-LDL receptor antibodies as in Fig. 3. Lane 1: PM from basal, potassium replete cells; lane 2: PM from basal, potassium depleted cells; lane 3: PM from insulin-stimulated, potassium replete cells; lane 4: PM from insulin-stimulated, potassium depleted cells; lane 5: LDM from basal, potassium replete cells; lane 6: LDM from basal, potassium depleted cells; lane 7: LDM from insulin-stimulated, potassium replete cells; lane 8: LDM from insulin-stimulated, potassium depleted cells.

In an attempt to further explore the action of insulin on LDL receptors, the effects of insulin on the subcellular distribution of LDL receptors were examined in adipocytes depleted of potassium (**Fig. 8**). Potassium depletion reversibly disrupts clathrin-coated pits and inhibits receptor-mediated endocytosis and cell polarization (42, 43). When isolated adipose cells were depleted of potassium, there was no increase in the number of LDL receptors found in the plasma membrane; the majority of LDL receptors were still found intracellularly. When adipose cells that had been potassium depleted were exposed to insulin, a marked decrease in the number of LDL receptors in the intracellular pool was again observed. However, in contrast to potassium replete cells where a decrease in the number of LDL receptors in the intracellular pool was seen without any significant changes in the number of LDL receptors in the plasma membrane, there was an increased appearance of LDL receptors in the plasma membrane of potassium depleted cells. Thus, when potassium is depleted, insulin seems to cause a reduction in LDL receptors from the intracellular pool by promoting a translocation to the plasma membrane.

To explore possible mechanisms for the insulin-induced decrease in LDL receptors in adipose cells, the effects of insulin on the rates of synthesis and degradation of LDL receptors were evaluated by following the incorporation of [^{35}S]methionine into immunoprecipitable LDL receptors (**Fig. 9**). Exposure of adipose cells to insulin caused a 33% increase in trichloroacetic acid-precipitable proteins (40401 ± 4459 vs. 53283 ± 6130 dpm/mg protein, $P <$ 0.001) and a 24% ($P < 0.001$) increase in LDL receptor synthesis. However, if the rate of LDL receptor synthesis is expressed relative to total protein synthesis, there were no significant effects of insulin. Interestingly, insulin decreased the half-life of pulse-labeled LDL receptors by ~40% from 4.8 ± 1.4 h in control cells to 2.9 ± 0.4 h in cells exposed to insulin (Fig. 9B). Thus, insulin appears to accelerate the degradation of LDL receptors in isolated rat adipose cells, thereby reducing the total number of receptors.

DISCUSSION

In the present studies we have used specific anti-LDL receptor antibodies to show that the LDL receptors present in rat adipose cells are immunologically similar to and of identical size as classical hepatic LDL receptors. However, the present results also provide a contrast to studies with fibroblasts and hepatocytes where other investigators have suggested that up to 90% of LDL receptors are localized to the cell surface and very few are

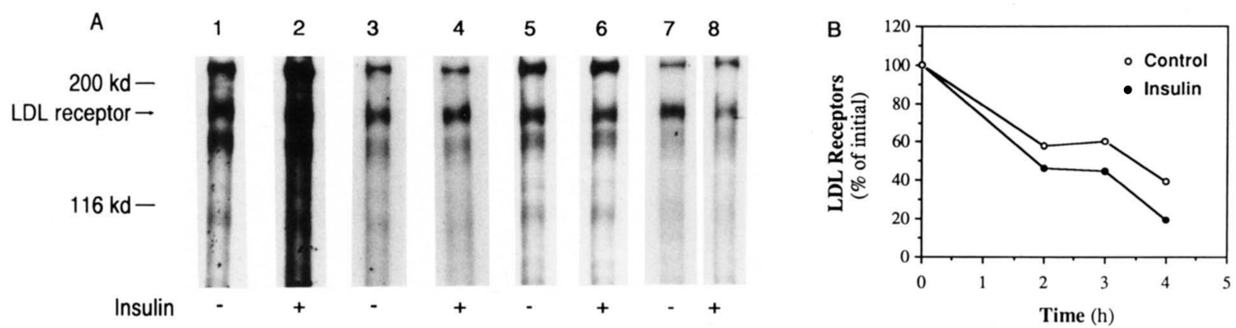

Fig. 9. Effects of insulin on the synthesis and degradation of LDL receptors in isolated adipose cells. Panel A: Autoradiograph of LDL receptors immunoprecipitated with anti-LDL receptor antibodies from adipose cells incubated with [^{35}S]methionine for 2 h and in the presence or absence of insulin (500 μU/ml). Cells were pulsed with [^{35}S]methionine for 2 h in the absence of insulin (lane 1) or in the presence of insulin (lane 2) before immunoprecipitation. Cells were pulsed with [^{35}S]methionine for 2 h in the absence of insulin, and chased with cold methionine for 2 h in the absence (lane 3) or presence of insulin (lane 4), for 3 h in the absence (lane 5) or presence of insulin (lane 6), or for 4 h in the absence (lane 7) or presence of insulin (lane 8) before immunoprecipitation. The autoradiograph was developed after 5 d exposure at −80°C. Panel B: Decline in immunoprecipitable LDL receptor radioactivity from adipose cells labeled with [^{35}S]methionine, chased with cold methionine in the absence or presence of insulin for the indicated times.

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present intracellularly (12). When rat adipose cells are separated into subcellular fractions by sucrose density gradient centrifugation, most of the LDL receptors are found in membranes of intracellular origin, with only 15–25% of the LDL receptors present in the plasma membrane. It is important to note, however, that while the subfractionation procedure is successful in enriching each subfraction with its marker enzyme, considerable cross contamination remains. After correcting the distribution of LDL receptors among the subfractions on the basis of enzyme recoveries (30), more than 70% of the LDL receptors are found in the LDM fraction associated primarily with enzyme markers of the Golgi complex. This distribution of LDL receptors is not a result of the anti-LDL receptor antibodies used to visualize the receptor as these antibodies do not recognize the VLDL receptor (see Fig. 2) nor due they recognize the LRP (34). In addition, binding studies of ^{125}I-labeled β-VLDL to the membrane subfractions yield similar results (data not shown); however, the binding data must be interpreted cautiously as they are probably less specific than the data generated with our anti-LDL receptor antibodies due to the potential confounding interaction of β-VLDL with the VLDL receptor and LRP. Moreover, the distribution of LDL receptors does not appear to be a function of cell isolation as it is observed not only in freshly isolated adipose cells, but also in adipose cells that have been cultured in lipoprotein-free medium to increase LDL receptor expression and, thus, allow for the regeneration of plasma membrane receptors that had possibly been destroyed during collagenase isolation of the cells, and in whole fat pads that have been subfractionated without prior treatment with collagenase to isolate cells.

Although the distribution of LDL receptors in adipose cells to locations that are primarily intracellular is somewhat unusual for a classical cell surface receptor, several observations support these conclusions. First, derivatization of cell surface receptors in fibroblasts or leukemia cells results in ~15–50% of cellular LDL receptors remaining unlabeled and, thus presumably located intracellularly (13). Second, derivatization of cell surface LDL receptors combined with subcellular fractionation of fibroblasts identifies only 39–49% of the receptors in the plasma membrane, with the remaining 50–60% localized to intracellular compartments (14). Third, increased amounts of LDL receptors are localized to intracellular vesicles in some tissues in transgenic mice overexpressing the human LDL receptor (15), although this distribution of receptors in transgenic animals could possibly be due to the abnormally high level of receptor expression. Fourth, the presence of a significant intracellular compartment of cell surface receptors appears to be a feature of other receptors and transport proteins such as asialoglycoprotein receptors (16), transferrin receptors (17), IGF II receptors (18), α_2-macroglobulin receptors (19), and glucose transporters (20). When subcellular fractionation techniques similar to those used in the present studies have been applied to these receptors and transporters, they have been localized primarily to the same LDM fraction associated with enzyme markers of the Golgi complex as observed in the present studies for the LDL receptor.

The functional significance of a large intracellular compartment of receptors is not fully understood, but such a compartment is likely to be composed of multiple pools of receptors. Some of these pools of receptors are undoubtedly in an endocytic-recycling pathway or in the process of being synthesized, while some receptors might possibly participate in the intracellular shuttling of proteins or lipids or represent a reservoir of receptors that could redistribute between the plasma membrane and the cell interior as a mechanism for rapidly regulating surface expression. Certainly, this latter possibility has been shown to be true for asialoglycoprotein receptors (22), transferrin receptors (22, 23), IGF II receptors (24), and α_2-macroglobulin receptors (25), as well as glucose transporters (26) when cells are exposed to insulin or phorbol esters. The earlier observation that ~60% of α_2-macroglobulin receptors/LRP are in the cell interior, with ~40% in the plasma membrane, in the basal state, and that insulin causes a 2- to 3-fold increase of receptors in the plasma membrane with a concomitant decrease in the intracellular compartment (25) is consistent with the recent observation that insulin increases the uptake of apolipoprotein E-enriched β-VLDL 2- to 3-fold in isolated rat adipocytes (44).

Other investigators have reported that exposure of cells to insulin increases the expression of LDL receptors in cultured fibroblasts (11), hepatocytes (27), and mononuclear cells (28); however, none of these cells are classical insulin-responsive tissues, at least as related to glucose transport, and the effects of insulin are observed only after 2–48 h of exposure to insulin. When isolated adipose cells are treated with insulin in the present experiments, insulin increases LDL receptor synthesis moderately, consistent with the insulin-induced increase in LDL receptor mRNA observed in hepatocytes (45); but this increase in LDL receptor synthesis appears to be a reflection of an insulin-induced increase in overall protein synthesis rather than a specific effect on LDL receptor synthesis. Indeed, treatment with insulin in the present experiments under standard conditions results in a 50% decrease in total LDL receptor number localized to intracellular compartments without any significant change in the number of LDL receptors in the plasma membrane. This decrease in LDL receptors with insulin appears to be due to an ~40% shortening of the half-life of [^{35}S]methionine-labeled LDL receptors. However, this degree of shortening of the half-life of LDL receptors (from ~5 h to ~3 h) cannot, by itself, fully explain the decrease in LDL recep-

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tors observed after a 30 min exposure to insulin. It is important to note that the half-life of LDL receptors was determined in experiments in which cells were pulse-labeled with [^{35}S]methionine for 2 h, conditions that do not label all the receptors within the cells. Thus, the discrepancy between the substantial and rapid decrease in immunoreactive LDL receptor mass and the modest shortening of the half-life of LDL receptors suggests that the LDL receptors within adipose cells exist in metabolically distinct pools. Consequently, it is suggested that there is a pool or pools of pre-formed LDL receptors that are preferentially degraded upon exposure to insulin while newly synthesized receptors constitute a pool of receptors that are not degraded rapidly. Therefore, the pulse-chase experiments, which were performed in whole cells and tracked total cell LDL receptors, are unable to measure the specific pool of receptors that are directed towards degradation after exposure to insulin and would be expected to underestimate the extent of the acceleration of the half-life of this specific pool of receptors that are rapidly degraded. Additional experiments will be required for direct proof of this hypothesis.

The net effect of the loss of intracellular LDL receptors induced by insulin is a decline in total cellular LDL receptors without any evidence for the redistribution of intracellular receptors to the plasma membrane. In contrast, insulin causes a marked translocation of glucose transporters from the LDM fraction to the plasma membrane under these same conditions, without altering the total number of cellular glucose transporters. Interestingly, when cells are depleted of potassium in order to disrupt the formation of clathrin-coated pits and inhibit receptor-mediated endocytosis (42, 46), insulin appears to increase the appearance of LDL receptors in the plasma membrane while decreasing the number of LDL receptors in the intracellular pool. Thus, under conditions where the endocytosis of clathrin-coated pits may be curtailed, a translocation of LDL receptors from the intracellular pool to the plasma membrane becomes apparent, similar to that seen for glucose transporters, IGF II receptors, and transferrin receptors. Because potassium depletion under basal conditions in the absence of insulin does not cause a discernible increase in LDL receptors in the plasma membrane, any constitutive movement of LDL receptors from the intracellular pool to the plasma membrane must proceed at a very slow rate, if at all.

Thus, the simplest explanation is that insulin appears to cause the sequential translocation of LDL receptors from an intracellular pool to the plasma membrane and then, if endocytosis of clathrin-coated pits is allowed to proceed normally, insulin directs internalized LDL receptors towards a degradative pathway. Although this explanation is consistent with the current data and with experiments on other membrane proteins that undergo translocation, other possibilities exist as potassium depletion can affect cell functions other than endocytosis. In particular, the effects of insulin on intracellular protein degradation appear to be partially due to intracellular fluxes of potassium (47). Thus, the insulin-induced accumulation of LDL receptors in the plasma membranes of potassium-depleted cells could be due to an impairment not only of endocytosis, but also of intracellular proteolysis. This more complicated explanation is supported by our preliminary observation that peptides derived from the $\alpha 1$ helix of the major histocompatibility complex class I antigens, which have been shown to inhibit the endocytosis of several recycling membrane proteins (48), accentuate the insulin-induced decrease in LDL receptors in isolated rat adipose cells without promoting their appearance in the plasma membranes (F. B. Kraemer, J. Stagsted, L. Olsson, and S. W. Cushman, unpublished observations). Therefore, insulin may cause LDL receptors to be either translocated to the plasma membrane and then degraded or directed to a degradative pathway without first undergoing translocation to the plasma membrane. Hence, the exact mechanisms responsible for the decrease in LDL receptors with insulin remain to be determined.

The net effect of insulin is to cause an overall decrease in LDL receptors in adipose cells primarily by post-transcriptional mechanisms mediated through an increased intracellular degradation of receptors. This post-transcriptional regulation of LDL receptor expression is different from most other systems where LDL receptor levels are determined by steady-state levels of LDL receptor mRNA which are, in turn, controlled by the rate of gene transcription (49, 50). However, congruent to the present results, thyrotropin has been reported to decrease LDL receptor number in a rat thyroid cell line without affecting LDL receptor mRNA levels (51), also suggesting non-transcriptional control. The reasons for the difference in the response of LDL receptors to insulin in adipose cells versus fibroblasts or hepatocytes are presently unknown, but could possibly be due to the fact that adipose cells are terminally differentiated while other cells where insulin has been reported to increase LDL receptors retain a capacity for cell division. In fact, when all the effects of insulin on LDL receptor expression in adipose cells are considered, there are both the expected, as well as unique events. The net result under normal conditions is an insulin-induced net decrease in total LDL receptors whether adipose cells are exposed to insulin for 5–30 min or 16 h.

While the role of the LDL receptor in adipose cells and why its regulation by insulin differs from other cells is unclear, a physiologic function of LDL receptors is suggested by the fact that exposure of rat adipose cells to LDL will inhibit cholesterol synthesis (52) while rates of cholesterol synthesis in these cells increase when serum cholesterol levels are pharmacologically lowered (53). This is further

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supported by our observation that the total number of LDL receptors in adipose cells increases during culture under lipoprotein-deficient conditions; however, no effects of culture on the relative distribution of receptors between the cell interior and the plasma membrane are observed. Therefore, although the LDL receptor in adipose cells appears to be regulated normally by cholesterol, its functional role is clouded by two recent findings. First, the LRP is found in adipose cells, can mediate the uptake of cholesterol esters from apolipoprotein E-enriched β-VLDL, and this uptake is increased by insulin (44). Second, the recently described VLDL receptor can bind VLDL, β-VLDL, and IDL with high affinity and is expressed in adipose tissue (40). Therefore, lipoprotein uptake by adipose cells could be mediated by either LDL receptors, LRP, or VLDL receptors, or any combination of these receptors. Several facts suggest that LDL receptors might not mediate lipoprotein uptake in adipose cells under all conditions. First, insulin decreases LDL receptors while increasing the translocation of LRP in adipose cells. Second, the insulin-stimulated uptake of apolipoprotein E-enriched β-VLDL in adipocytes is entirely blocked either by antibodies to LRP or by the 39-kDa α_2-macroglobulin receptor-related protein (44), which copurifies with LRP and appears to modulate the binding of ligands to the LRP (54): findings that are consistent with uptake occurring via LRP and not via the LDL receptor. Thus, it is possible that LDL receptors might exert a different function in adipocytes and this function, as well as any unusual regulation, might relate to unique aspects of cholesterol metabolism in adipose tissue.

Adipose tissue is a major storage site of cholesterol, accounting for 15-20% of body cholesterol stores in normal individuals and even greater percentages in obesity (55). As opposed to many other cell types, up to 90% of the cholesterol in adipose cells is unesterified (56, 57). Furthermore, this unesterified cholesterol is not predominantly found in cellular membranes, but is localized within the central lipid droplet of the adipose cell. This unique feature probably accounts for the finding that the larger the size of the cell, the greater the amounts of cellular cholesterol present (52, 55, 56); presumably, the larger oil droplet is able to accommodate more unesterified cholesterol. Isotopic studies of cholesterol turnover have substantiated that adipose cell cholesterol is in dynamic equilibrium with plasma cholesterol, but constitutes only a slowly turning over pool of total body stores (57, 58). How these aspects of cholesterol metabolism relate to LDL receptor regulation and function in adipose tissue remains to be determined, but might have important implications for abnormalities of cholesterol metabolism found to be associated with obesity.

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