Isoproterenol decreases LDL receptor expression in rat adipose cells: activation of cyclic AMP-dependent proteolysis

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Abstract The low density lipoprotein (LDL) receptor is part of a family of proteins that mediate the uptake of lipoproteins into cells. In this paper we have demonstrated the over-expression in E. coli of a rat LDL receptor fusion protein that contains the region of the receptor sharing homology with the EGF precursor. The fusion protein was utilized to immunize rabbits and successfully generate antibodies that recognize the intact LDL receptor. These anti-LDL receptor/fusion protein antibodies were used to examine the effects of cyclic AMP on the expression of LDL receptors in isolated rat adipocytes. Incubation of adipocytes with isoproterenol caused a dose-dependent diminution in intact LDL receptors and a smaller immunoreactive proteins. Pulse-chase experiments demonstrated that isoproterenol rapidly shortened the initial half-life of intact, immunoprecipitable LDL receptors in the plasma membrane. The effects of isoproterenol on LDL receptor expression were mimicked by forskolin, by an analog of cyclic AMP, and by ACTH. In contrast, incubation with propranolol blocked the effects of isoproterenol on LDL receptor expression. While antioxidants and several different protease inhibitors had no effects, N-acetyl-leucine-leucine-methionine (ALLM) was able to prevent the isoproterenol-induced effects on LDL receptors. Thus, it appears that agents acting via cyclic AMP cause a rapid decrease in LDL receptors in the plasma membranes of isolated adipose cells due to the apparent stimulation of an ALLM-sensitive protease that degrades the LDL receptor. These results suggest a novel mechanism for the posttranscriptional regulation of LDL receptor expression in adipocytes. —Kraemer, F. B., V. Natu, A. Singh-Bist, S. Patel, M. C. Komaromy, S. Medicherla, S. Azhar, and C. Sztalryd. Isoproterenol decreases LDL receptor expression in rat adipose cells: activation of cyclic AMP-dependent proteolysis. J. Lipid Res. 1996. 37: 237–249.

Supplementary key words LDL receptor • fusion protein • recombinant protein • expression in bacteria • immunoblot • mRNA • cyclic AMP • rat

The low density lipoprotein (LDL) receptor is a member of a class of surface proteins that are able to recognize and bind apolipoprotein (apo) E- and apoB-containing lipoproteins, with subsequent receptor-mediated uptake of the lipoprotein (1). The LDL receptor is expressed in almost all tissues, including adipose tissue, and the number of LDL receptors expressed by a cell is regulated primarily by cellular sterol content, with sterols inhibiting the transcription of LDL receptor mRNA through the interaction of intermediate effectors (2) with sterol regulatory elements in the LDL receptor gene (3). In addition to sterols, LDL receptor expression is regulated by growth factors, such as platelet-derived growth factor (4), and hormones, such as thyroid hormone (5) and estrogen (6), although regulation by estrogen seems to be indirect and to be mediated by growth hormone (7). Several other hormones have been examined for their effects on LDL receptor expression. Insulin has been reported to increase LDL receptors in fibroblasts (4), mononuclear leukocytes (8), and hepatocytes (9); however, we recently reported that in vitro treatment of rat adipocytes with insulin caused a rapid decrease in LDL receptors in this tissue (10). Catecholamines and cyclic AMP have been reported to decrease LDL receptors in fibroblasts, hepatocytes, and monocytes (8, 11, 12), while thyrotropin, acting through cyclic AMP, decreases LDL receptor expression in nontransformed thyroid cells (13). However, other investigators have found an increase in LDL receptors in hepatocytes treated with catecholamines, glucagon, and cyclic AMP (14). Other hormones acting through cyclic AMP, such
as ACTH and LH, cause an increase in LDL receptors in steroidogenic tissues, such as adrenal (15) and ovary (16), although it appears that these effects might be mediated through changes in intracellular sterols, i.e., depletion of regulatory pools of sterols, rather than via direct actions of cyclic AMP (17).

Because adipose tissue is excessively sensitive to the metabolic changes that occur with feeding and fasting, we recently examined the effects of short term fasting in rats on the expression of LDL receptors in adipose cells (18). We observed that 2 days of food deprivation caused a 95% reduction in LDL receptor protein that was due, in part, to a decline in the levels of LDL receptor mRNA and in the rate of synthesis of LDL receptors; however, additional posttranslational mechanisms appeared to contribute to the diminished expression of LDL receptors. As fasting is associated with an increase in counter-regulatory hormones, many of whose actions are mediated via cyclic AMP (catecholamines, ACTH, glucagon), we examined the effects of these agents and cyclic AMP on the expression of LDL receptors in isolated rat adipose cells. The results of the current studies show that agents acting via cyclic AMP cause a rapid decrease in LDL receptors in the plasma membranes of isolated adipose cells due to the apparent stimulation of an N-acetyl-leucine-leucine-methionine (ALLM)-sensitive protease that degrades the LDL receptor.

METHODS

Chemicals

Reagents were obtained from the following sources: collagenase (Worthington Biochemical Corp., Freehold, NJ); Triton X-100, leupeptin, aprotinin, (Sigma Chemical Co., St. Louis, MO); cyclic 8-(4-chlorophenylthio)adenosine-3':5' monophosphate (Boehringer Mannheim Biochemicals, Indianapolis, IN); bovine serum albumin (Intergen Co., Purchase, NY); ECL western blotting detection reagents, horseradish peroxidase-linked whole antibody anti-rabbit IgG (Amersham Life Sciences Products, Arlington Heights, IL); nitrocellulose paper (Schleicher and Schuell, Keene, NH). All other chemicals were obtained from standard commercial sources.

Animals

Male Sprague-Dawley rats (180–240 g, B and K, Fremont, CA) were obtained and maintained on ad lib rat chow and tap water with a 12 h light/dark cycle according to Stanford University guidelines. Some animals were treated with 17α-ethinyl estradiol (10 mg/kg) subcutaneously for 3 days prior to killing (19). Animals were killed by decapitation and epididymal fat pads were quickly removed from each rat and washed with phosphate-buffered saline (pH 7.4) for isolation of adipocytes. Other tissues were immediately removed, frozen in liquid nitrogen and stored at -80°C prior to being used for studies. Antibodies were produced in white New Zealand rabbits (RR Rabbity, Stanwood, WA) that were maintained on ad lib rabbit chow and tap water according to Stanford University guidelines.

Adipose cell isolation and membrane preparation

Adipose cells were isolated by collagenase digestion under sterile techniques as previously described (10). Collagenase digestion was carried out in Krebs-Ringer-bicarbonate-HEPES (KR BH) buffer (pH 7.4) containing 5% bovine serum albumin (BSA), and 200 nM adenosine. After washing, the isolated adipose cells were incubated in KR BH buffer with BSA and adenosine in an atmosphere of 95% air/5% CO2 in a shaking (60 cycles/min) water bath at 37°C. 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. An aliquot was taken for protein determination and the cell homogenates were fractionated by differential ultracentrifugation to yield plasma membranes (PM) and intracellular membranes (IM), as described previously (10). In some instances total membranes were prepared by centrifugation at 100,000 g for 60 min in a Beckman 70 Ti rotor.

Fusion protein and antibody production

The fusion protein was partially purified on SDS-PAGE and encoded by the vector. The LDLR expression vector was transformed into E. coli strain BL21(DE3)LysS, which contains T7 polymerase under the control of the lac UV5 promoter (23). After induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside, large amounts of the expected 28,896 dalton fusion protein were produced. The fusion protein was partially purified on SDS-PAGE gels, excised, and homogenized in phosphate-buffered saline. Yields of fusion proteins varied between 4 and 150 mg per preparation, depending on the amount of bacteria grown. The fusion proteins were emulsified in Ribi Adjuvant System® (RIBI Immunochem Research, Inc., Helena, MT) and rabbits were immunized by intradermal, intramuscular, subcutaneous, and intraperitoneal injections. Animals were boosted monthly and bled 10 days after each immunization. IgG was purified by chromatography on protein A-Sepharose (20).

**Immunoblotting**

Membrane fractions were homogenized in buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.26 M sucrose, 1% Triton X-100, 2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml PMSF. Samples were electrophoresed under reducing (1% β-mercaptoethanol) or nonreducing conditions on 8% polyacrylamide gels containing 0.1% SDS after the addition of 0.5% SDS and 13% glycerol to the samples, as described previously (24). E. coli were extracted either directly in 20 mM Tris-HCl (pH 7.4), 0.5% SDS, and 13% glycerol (sample buffer), or lysed in H2O followed by centrifugation at 10,000 × g for 10 min and solubilization of the H2O-insoluble pellet in extraction buffer. After electrophoresis, the proteins were transferred to nitrocellulose paper. The nitrocellulose paper was incubated at 37°C for 3 h with blocking buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 3% Carnation® Instant Milk. Incubation buffer was drained and the nitrocellulose was incubated with fresh blocking buffer containing rabbit polyclonal anti-LDLR/fusion protein antibodies at a final IgG concentration of 0.1–1 μg/ml for 12 h at 4°C. After the addition of Pansorbin for 60 min, the immune complexes were isolated by centrifugation. The pellets were washed in buffer containing 0.1 mM Tris-HCl and 0.05 mM LiCl and in buffer containing 0.1% lauryl sarcosyl, dissolved in sample buffer and then electrophoresed on SDS polyacrylamide gels as above. After drying, the gels were visualized in a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA).

**Immunoprecipitation**

Isolated adipose cells were incubated at 37°C under an atmosphere of 95% air/5% CO2 in Dulbecco’s modified essential media (DMEM) deficient in methionine containing 3% BSA and 100 μCi/ml of [35S]methionine (Amersham Life Sciences Products, Arlington Heights, IL). After incubation for 2 h, the cells were washed once with DMEM–9% BSA supplemented with 3 mM cold methionine and then incubated in fresh media in the absence or presence of 10−7 M isoproterenol for various times (10). At the end of the incubations, cells were rapidly separated from the medium by centrifugation in a microfuge through 0.5 ml of silicone oil. Packed cells were collected and plasma membranes were isolated as described above. PM were solubilized in 0.15 M NaCl, 9% Triton X-100, 0.1% lauryl sarcosyl, 1 mM PMSF, 1 unit/ml leupeptin, and 0.2 mg/ml aprotinin, and then incubated with rabbit polyclonal anti-LDLR/fusion protein antibodies at a final IgG concentration of ~0.5 μg/ml for 12 h at 4°C. After the addition of Pansorbin for 60 min, the immune complexes were isolated by centrifugation. The pellets were washed in buffer containing 0.1 mM Tris-HCl and 0.05 mM LiCl and in buffer containing 0.1% lauryl sarcosyl, dissolved in sample buffer and then electrophoresed on SDS polyacrylamide gels as above. After drying, the gels were visualized in a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA).
RNAse protection assay

The concentration of LDL receptor mRNA was determined using a sensitive RNAse protection assay as described previously (25, 26). A 1.2 kb fragment, generated by polymerase chain reaction from a rat LDL receptor cDNA and representing nucleotides 1878 through the 3' untranslated region (22), was subcloned into the EcoRI site of pBluescript KS II(+) (Stratagene, La Jolla, CA). Similarly, a 274 bp Dra II-Tha I fragment (position 134-408) of 18S ribosomal RNA cDNA (27) was subcloned into the Dra I-Eco RV site of pBluescript KS II(+) (Stratagene, La Jolla, CA). The plasmids were linearized with appropriate restriction endonucleases (Xho I for LDL receptor and Bam HI for 18S ribosomal RNA). The antisense cRNA probes were synthesized using [32P]rCTP and either T7 or T3 RNA polymerase following the method supplied in the Stratagene in vitro transcription kit. Total cellular RNA was isolated from adipose cells by CHCl₃-phenol extraction (18). Aliquots of total RNA (10-20 μg) or control tRNA (10-20 μg) were hybridized with freshly synthesized antisense LDL receptor or 18S ribosomal RNA cRNA transcripts and the RNA:RNA hybrids were digested with RNase A and T₁. The protected fragments (284 nt and 274 nt for LDL receptor and 18S ribosomal RNA, respectively) were resolved by electrophoresis followed by autoradiography and analyzed by laser densitometry as described above.

Other assays

Protein was measured with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL).

RESULTS

Fusion protein generation and antibody production

As production of antibodies against rat hepatic LDL receptors (19) can be tedious due to the need to purify large amounts of the receptor, we sought an alternative method for generating anti-LDL receptor antibodies that relies on expressing a portion of the receptor as a bacterial fusion protein. In order to develop an LDL receptor fusion protein that could be expressed in large quantities, a 675 base portion, nucleotides 1636-2310 (LDLR-2), of rat LDL receptor was subcloned into a PET expression vector under the control of T7 RNA polymerase. LDLR-2 encompasses a region of the LDL receptor that is similar to the EGF precursor (28) and contains a single class B cysteine-rich motif (repeat 3) (29). The LDLR expression vector was transformed into E. coli strain BL21(DE3)LyS and selected by ampicillin resistance. Single colonies were expanded and then incubated without (lane 1) or with (lane 2) 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. The E. coli were lysed and extracts were subjected to SDS-PAGE, fixed, and stained with Coomassie G250. Panel B: Extracts of transformed E. coli were immunoblotted with anti-fusion protein IgG (lane 1) or with rabbit pre-immune IgG (lane 2), as described in the Methods.

Fig. 1. Expression of LDLR/fusion protein in E. coli (A) and recognition by polyclonal antibodies (B): Panel A: A 675 base portion, nucleotides 1636-2310 (LDLR-2), of rat LDL receptor was subcloned into a PET expression vector under the control of T7 RNA polymerase. The LDL receptor expression vector was transformed into E. coli strain BL21(DE3)LyS and selected by ampicillin resistance. Single colonies were expanded and then incubated without (lane 1) or with (lane 2) 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. The E. coli were lysed and extracts were subjected to SDS-PAGE, fixed, and stained with Coomassie G250. Panel B: Extracts of transformed E. coli were immunoblotted with anti-fusion protein IgG (lane 1) or with rabbit pre-immune IgG (lane 2), as described in the Methods.

Fig. 2. Immunoblot of LDL receptor in rat tissues. Extracts (50 μg) of normal rat liver membranes (lane 1), extracts (50 μg) of estradiol-treated rat liver membranes (lane 2), and extracts (100 μg) of rat epididymal fat (lane 3) were electrophoresed on SDS-PAGE, transferred to nitrocellulose filters, immunoblotted with anti-LDLR/fusion protein IgG (1:5,000 dilution), and visualized by enhanced chemiluminescence.
induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside, large amounts of the expected ~29 kDa fusion protein were produced (Fig. 1A). The fusion protein was partially purified on SDS-PAGE gels, excised, and used to immunize rabbits. The antibodies generated recognized the LDLR/fusion protein solubilized from transformed E. coli (Fig. 1B), as well as several minor bacterial proteins of both higher and lower molecular weight when the films were overexposed. Preimmune serum or IgG did not recognize the LDLR/fusion proteins, although some preparations did show reaction with similar higher and lower molecular weight bacterial proteins when the films were overexposed.

In order to determine whether the anti-LDLR/fusion protein antibodies could recognize intact LDL receptors, membranes of livers from normal and estrogen-treated rats, as well as from rat epididymal fat, were solubilized and immunoblotted with anti-LDLR/fusion protein IgG (Fig. 2). In comparison with the ~29 kDa fusion protein recognized in extracts of E. coli, the antibodies reacted with a prominent protein of ~135 kDa, which corresponds to the size of intact LDL receptor, in each of the extracts. In addition to the ~135 kDa protein detected by the antibodies, a protein of ~110 kDa was observed that presumably represents the LDL receptor precursor, and a prominent protein of ~50–55 kDa was also observed that apparently represents a nonspecific band as it is seen on all blots and the intensity of the band does not vary among different tissues or with various manipulations. As previously reported (6, 19), approximately 10-fold more LDL receptor protein could be seen in membranes of livers from estrogen-treated rats compared to normal controls.

To explore further the specificity of the antibodies for the LDL receptor, membranes of livers from estrogen-treated rats were electrophoresed and transferred to nitrocellulose. The filters were immunoblotted with anti-LDLR/fusion protein IgG in the presence of increasing amounts of LDLR/fusion protein (Fig. 3). Incubation with LDLR/fusion protein diminished the recognition of intact LDL receptors, while a rat lipoprotein lipase/fusion protein produced with the same expression system had no effect. These data further support the conclusion that the ~135 kDa protein in liver and epididymal fat recognized by the anti-LDLR/fusion protein antibodies is the LDL receptor.

Recently, an LDL receptor-like protein, termed the VLDL receptor, that binds VLDL, β-VLDL and IDL, but not LDL, with high affinity, has been cloned from rabbits, humans, and mice (30–32). The VLDL receptor cDNA encodes a protein of similar size to the LDL receptor and shares ~50% homology with the LDL receptor, including the region of our LDL fusion protein; however, as opposed to the LDL receptor, VLDL receptor mRNA is predominantly found in muscle and
adipose tissues, with barely detectable amounts in liver.

In order to explore the specificity of our anti-LDLR/fusion protein antibodies further, we examined whether our anti-LDLR/fusion protein antibodies recognized the VLDL receptor. Extracts from adipose tissue of control mice and mice in which functional LDL receptors had been removed by homologous recombination (33) were immunoblotted with anti-LDLR/fusion protein antibodies (Fig. 4). Immunoreactive LDL receptors were seen in adipose tissue of control mice, while no specific immunoreactive proteins were observed in adipose tissue from LDL receptor knockout mice. However, in addition to the nonspecific protein observed at ~55 kDa, there was a protein observed at ~100 kDa in both control and LDL receptor knockout mice that was not seen in rats. Thus, our polyclonal antibody raised against a rat LDL receptor fusion protein appears to be specific for LDL receptors and does not recognize the VLDL receptor in mice.

Isoproterenol

Because catecholamines are known to exert a major effect on adipose cells, the effects of acute exposure to isoproterenol on the level of LDL receptor expression and its distribution in adipose cells were examined. Isolated adipose cells were exposed to isoproterenol for 30 min, subfractionated, and immunoblotted for LDL receptors (Fig. 5). As reported previously (10), LDL receptors were found in plasma and intracellular membranes in control adipose cells, but LDL receptors were enriched in intracellular membranes, primarily representing membranes of the Golgi complex. When isolated adipose cells were exposed to isoproterenol (10^5 M) for 30 min, no discernible changes in the amount of LDL receptor in intracellular membranes were observed; however, intact LDL receptors were reduced in the plasma membranes and were associated with the appearance of a new immunoreactive protein that ran

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**Fig. 5.** Effects of isoproterenol on LDL receptor expression in isolated rat adipose cells. Panel A: Autoradiograph of an immunoblot of LDL receptors in intracellular (IM) and plasma (PM) membranes prepared from isolated adipose cells incubated in the presence or absence (basal) of isoproterenol (10^5 M) for 30 min. IM and PM were prepared by sucrose gradient centrifugation from adipose cells isolated from rat epididymal fat pads. Membrane fractions (50 μg each) were separated on SDS-PAGE, transferred to nitrocellulose, incubated with rabbit polyclonal anti-LDLR/fusion protein IgG (1:1,000 dilution), and visualized by enhanced chemiluminescence as described in Materials and Methods. The autoradiograph was developed after 5 sec. Lane 1: IM from basal adipose cells; lane 2: IM from adipose cells treated with isoproterenol; lane 3: PM from basal adipose cells; lane 4: PM from adipose cells treated with isoproterenol. Panel B: Autoradiograph of an immunoblot of LDL receptors in PM prepared from isolated adipose cells incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of isoproterenol (10^5 M) for 30 min. PM were prepared, and fractions (50 μg, lanes 1 and 2; 100 μg, lanes 3 and 4) were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with anti-LDLR/fusion protein IgG as above. The autoradiograph was developed after 2 min. Panel C: Autoradiograph of an immunoblot of LDL receptors in PM prepared from isolated adipose cells incubated in the absence (basal) or presence of the indicated concentrations of isoproterenol (iso) for 30 min. PM were prepared, and fractions (50 μg each) were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with anti-LDLR/fusion protein IgG as above. The autoradiograph was developed after 5 sec.
A. 12345
B. 200k - LDLReceptor ~ 97 kd - 66 kd - 116 kd - 45 kd

Chase Isoproterenol

Fig. 6. Effects of isoproterenol on the degradation of LDL receptors in isolated adipose cells. Panel A: Autoradiograph of LDL receptors immunoprecipitated with anti-LDLR/fusion protein IgG from plasma membranes of adipose cells incubated with [35S]methionine for 2 h in the absence of isoproterenol (lane 1) and chased with cold methionine for 30 min in the absence (lane 2) or presence (lane 3) of 10^7 m isoproterenol, or chased with cold methionine for 60 min in the absence (lane 4) or presence (lane 5) of 10^7 m isoproterenol. Plasma membranes were isolated and immunoprecipitation was performed as described in the Materials and Methods. Panel B: Densitometric scan of the decline in immunoprecipitable LDL receptors from adipose cells labeled with [35S]methionine and chased with cold methionine in the absence (control) or presence of isoproterenol for the indicated times.

as a doublet on some gels and as a single band on other gels at ~90-95 kDa (Fig. 5A), suggesting that a proteolytic product(s) was generated. Figure 5B displays a separate experiment in which two different concentrations of plasma membranes from adipose cells incubated in the absence or presence of isoproterenol have been immunoblotted for LDL receptors, allowing better visualization and quantitation of the reduction in intact LDL receptors after exposure to isoproterenol. Isoproterenol caused an ~95% decrease in intact LDL receptors in this experiment that was associated with the appearance of an apparent ~90-95 kDa proteolytic product, as well as several smaller molecular weight proteins, one or more of which appear to coincide with the 50-55 kDa “nonspecific” band. In order to explore whether the isoproterenol-induced disappearance of intact LDL receptors from plasma membranes and the appearance of a proteolytic product were dose dependent, isolated adipose cells were incubated with varying concentrations of isoproterenol for 30 min (Fig. 5C). The ~90-95 kDa proteolytic product began to appear at 2 nM isoproterenol and a maximum effect was observed by 100 nM. The concentrations of isoproterenol that cause the disappearance of intact LDL receptors and the appearance of an apparent proteolytic product thus parallel the affinity of isoproterenol for the β-adrenergic receptor (34).

The rapid disappearance of intact LDL receptors from the plasma membrane after exposure to isoproterenol suggested that the rate of degradation of LDL receptors was accelerated. To examine this directly, the rate of degradation of LDL receptors in the plasma membrane was evaluated by following the incorporation of [35S]methionine into immunoprecipitable LDL receptors (Fig. 6). Exposure of isolated adipose cells to isoproterenol rapidly decreased the amount of radiolabeled, immunoprecipitable, intact LDL receptors in the plasma membrane (Fig. 6A). If these changes in the initial degradation of LDL receptors observed after 30 and 60 min of exposure to isoproterenol are extrapolated, the half-life of pulse-labeled, intact LDL receptors in the plasma membrane decreased ~7-fold from a half-life of 16.4 h in control to 2.25 h in cells exposed to isoproterenol (Fig. 6B). Lower molecular weight fragments of the LDL receptor were not detected in these immunoprecipitation experiments. Thus, isoproterenol appears to accelerate the initial rate of degradation of intact LDL receptors from the plasma membranes of isolated adipose cells.

In order to determine whether the changes observed in the LDL receptor were due to isoproterenol stimulation of adenylate cyclase, isolated adipose cells were incubated with forskolin, which directly activates adenylate cyclase, for 30 min, subfractionated, and immunoblotted for LDL receptors. As shown in Fig. 7A, forskolin did not cause any changes in LDL receptors detected in intracellular membranes, but did cause the disappearance of intact LDL receptors and the appear-
Fig. 7. Effects of forskolin, an analog of cyclic AMP, and ACTH on LDL receptor expression in isolated rat adipose cells. Panel A: Autoradiograph of an immunoblot of LDL receptors in intracellular (IM) and plasma (PM) membranes prepared from isolated adipose cells incubated in the presence or absence (basal) of forskolin (10^{-5} M) for 30 min. IM and PM were prepared, and fractions (50 µg each) were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with anti-LDLR/fusion protein IgG as in Fig. 5. The autoradiograph was developed after 5 sec. Lane 1: IM from basal adipose cells; lane 2: IM from adipose cells treated with forskolin; lane 3: PM from basal adipose cells; lane 4: PM from adipose cells treated with forskolin. Panel B: Autoradiograph of an immunoblot of LDL receptors in PM prepared from isolated adipose cells incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of forskolin (10^{-5} M) for 30 min. PM were prepared, and fractions (50 µg, lanes 1 and 2; 100 µg, lanes 3 and 4) were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with anti-LDLR/fusion protein IgG as above. The autoradiograph was developed after 2 min. Panel C: Autoradiograph of an immunoblot of LDL receptors in PM prepared from isolated adipose cells incubated in the absence (basal) or presence of forskolin, cyclic 8-(4-chlorphenylthio)-AMP (cAMP), or ACTH for 30 min. The autoradiograph was developed after 5 sec. Lane 1: PM from basal adipose cells; lane 2: PM from adipose cells treated with forskolin (10^{-5} M); lane 3: PM from adipose cells treated with cyclic 8-(4-chlorphenylthio)-AMP (10^{-3} M); lane 4: PM from adipose cells treated with ACTH (10^{-5} M); lane 5: PM from adipose cells treated with ACTH (10^{-4} M); lane 6: PM from adipose cells treated with ACTH (10^{-3} M).

The appearance of an apparent ~90–95 kDa proteolytic product from plasma membranes, as seen with isoproterenol. Figure 7B displays a separate experiment in which two different concentrations of plasma membranes from adipose cells incubated in the absence or presence of forskolin have been immunoblotted for LDL receptors, allowing better visualization and quantitation of the reduction in intact LDL receptors after exposure to forskolin. Similar to the observation with isoproterenol, forskolin caused an ~85% decrease in intact LDL receptors in this experiment that was associated with the appearance of an apparent ~90–95 kDa proteolytic product. These results suggest that the isoproterenol-induced decrease in intact LDL receptors and the appearance of an apparent proteolytic product in plasma membranes is mediated via generation of cyclic AMP, rather than a potential nonspecific effect of isoproterenol on the cell membrane. To explore this further, isolated adipose cells were incubated with a nondegradable analog of cyclic AMP or with various concentrations of ACTH (Fig. 7C). As observed for isoproterenol and forskolin, the cyclic AMP analog caused a decrease in intact LDL receptors and the appearance of an apparent proteolytic product in plasma membranes. In addition, ACTH, which binds to a different receptor but acts via cyclic AMP, also decreased intact LDL receptors and caused the appearance of an apparent proteolytic product in plasma membranes in a dose-dependent manner. Thus, it appears that the generation of cyclic AMP within the adipose cell causes a decrease in LDL receptors in the plasma membrane.

As another means of documenting that the observed effects were not due to a nonspecific action of these agents or carriers on the plasma membrane, isolated adipose cells were incubated with isoproterenol in the presence of increasing amounts of a β-adrenergic recep-
Fig. 8. Ability of propranolol to block the effects of isoproterenol on LDL receptors in isolated rat adipose cells. Autoradiograph of an immunoblot of LDL receptors in plasma membranes (PM) prepared from isolated adipose cells incubated in the absence (basal) or presence of isoproterenol (iso) and propranolol (prop) for 30 min. PM were prepared, and fractions (50 μg each) were separated on SDS-PAGE, transferred to nitrocellulose, and incubated with anti-LDL/fusion protein IgG as in Fig. 5. Lane 1: PM from basal adipose cells; lane 2: PM from adipose cells treated with isoproterenol (10^{-7} M); lane 3: PM from adipose cells treated with isoproterenol (10^{-7} M) and propranolol (10^{-5} M); lane 4: PM from adipose cells treated with isoproterenol (10^{-7} M) and propranolol (10^{-6} M).

ator antagonist, propranolol (Fig. 8). While 100 nM isoproterenol caused a decrease in intact LDL receptors and the appearance of an apparent proteolytic product, propranolol blocked these effects. Thus, the actions of isoproterenol, and presumably the other agents utilized, on LDL receptors in adipose cells were receptor mediated. Although the effects on LDL receptors appear to be receptor mediated and dependent on cyclic AMP as a second messenger, the primary action in adipose cells of isoproterenol, ACTH, and cyclic AMP is to stimulate lipolysis. In order to examine whether the release of free fatty acids contributed to the observed changes in LDL receptor expression, isolated adipose cells were incubated in the presence of 1 mM oleate for 30 min (Fig. 9). No effects on LDL receptors were observed. In addition, to explore the possibility that the release of free fatty acids was associated with an oxidative stress that might have caused the apparent proteolysis of the LDL receptors in the plasma membranes, isolated adipose cells were incubated with isoproterenol in the presence of butylated hydroxytoluene (BHT). Isoproterenol caused a decrease in intact LDL receptors and the appearance of an apparent proteolytic product both in the absence and presence of BHT (Fig. 9). Thus, it does not appear that the proteolysis of LDL receptors induced by isoproterenol is caused by the fatty acids released from adipose cells during lipolysis or by BHT-sensitive lipid oxidation associated with lipolysis.

As the effects on LDL receptors in adipose cells of isoproterenol and the other agents that act through cyclic AMP were rapid and were associated with the appearance of a new immunoreactive protein that was smaller than intact LDL receptors, it was possible that the changes observed were due to the activation of a cellular protease. In an attempt to determine whether cellular proteases might be participating in this phenomenon, several different protease inhibitors were co-incubated with isoproterenol and the effects on the appearance of the new immunoreactive protein were examined. Incubation with EDTA (0.005 M), antipain (200 μg/ml), bestatin (200 μg/ml), or chymostatin (200 μg/ml) failed to prevent the isoproterenol-induced decrease in intact LDL receptors and the appearance of the apparent proteolytic product (data not shown). However, incubation with N-acetyl-leucine-leucine-methionine (ALLM), a cysteine protease inhibitor, blocked the effects of isoproterenol on the appearance of LDL receptors.
Because isoproterenol and other agents acting through cyclic AMP could affect LDL receptor expression by other mechanisms in addition to accelerating the degradation of intact receptors in the plasma membrane by activation of an apparent ALLM-sensitive protease, the effects of isoproterenol on the expression of steady-state levels of LDL receptor mRNA were examined (Fig. 11). Isolated adipose cells were incubated in the absence or presence of $10^{-7}$ M isoproterenol for 30 min and the level of LDL receptor mRNA was determined by an RNase protection assay. Isoproterenol caused ~20% decrease in the level of LDL receptor mRNA without any changes observed in ribosomal 18S mRNA.

DISCUSSION

Using antibodies generated against a rat LDL receptor/fusion protein that recognize intact LDL receptors on immunoblots, we have demonstrated that isoproterenol and other agents acting through cyclic AMP rapidly decrease LDL receptor expression in plasma membranes of isolated rat adipocytes. Associated with this loss of intact LDL receptors is the appearance of smaller LDL receptor immunoreactive proteins, possibly due to the rapid proteolysis of the LDL receptor. The loss of intact LDL receptors and the appearance of proteolytic fragments within the plasma membrane of the isolated adipocytes occurred rapidly after exposure to isoproterenol, achieving a maximum effect by 30 min. In pulse-chase experiments, isoproterenol appeared to accelerate the initial rate of degradation of intact LDL receptors from the plasma membranes of isolated adipose cells; however, the rapid, profound loss of LDL receptors is probably due to a combination of these changes in the degradation of the receptor along with a...
decrease in the level of LDL receptor mRNA observed after exposure to isoproterenol. These effects of isoproterenol do not appear to be the result of nonspecific actions on the LDL receptor within the membrane as 1) the effects were mimicked by ACTH, forskolin, and a cyclic AMP analog; 2) the effects occurred only with LDL receptors in the plasma membrane and not with LDL receptors found in intracellular membranes; 3) several different protease inhibitors (EDTA, aprotinin, leupeptin, and PMSF) were present in the buffers used during adipocyte homogenization and membrane preparation; and 4) the effects were not observed when adipocytes were homogenized and membrane fractions were prepared prior to exposure to isoproterenol (data not shown). In addition, propranolol, a beta-adrenergic receptor antagonist, blocked the ability of isoproterenol both to decrease intact LDL receptors and to cause the appearance of proteolytic fragments of the LDL receptor in the plasma membrane, further supporting that the effects were specific and mediated via generation of cyclic AMP. As generation of cyclic AMP causes the activation of lipolysis in adipocytes, it was possible that lipolytic products or oxidant stress could have been responsible for the apparent isoproterenol stimulation of LDL receptor proteolysis. This does not appear to be the case as incubation of isolated adipose cells with oleate had no effects on the expression of the LDL receptor. This is in contrast to a recent report that oleate increases LDL receptors in fibroblasts, macrophages, and hepatocytes (35); however, this observed increase was seen only after several hours of treatment, as opposed to the 90-min incubation in the current studies. Furthermore, co-incubation of antioxidants had no effects on the isoproterenol-mediated degradation of LDL receptors in the plasma membrane, suggesting that generation of oxygen free radicals was not involved in the apparent proteolysis of the LDL receptor in the plasma membranes.

In an attempt to further characterize the apparent isoproterenol-stimulated proteolysis of LDL receptors, incubations with various protease inhibitors were carried out. Only ALLM, an inhibitor of cysteine proteases such as cathepsin L, cathepsin B, cathepsin D, and calpains (36), prevented the isoproterenol-stimulated proteolysis of LDL receptors. The metalloprotease inhibitor EDTA, the amino peptidase inhibitor bestatin, the chymotrypsin inhibitor chymostatin, and the serine and cysteine protease inhibitor antipain, were all ineffective; however, it is possible that differences in the ability of these compounds to penetrate the cell might have contributed to some of the apparent ineffectiveness. Interestingly, proteases that are inhibited by ALLM and its closely related compound, ALLN (N-acetyl-leucyl-leucyl-norleucinal), have been implicated in the degradation of several proteins involved in cellular cholesterol metabolism, including HMG-CoA reductase (36), apolipoprotein B (37), and SREBP-1 (2). The identities of the proteases responsible for the degradation of these proteins and for the apparent proteolysis of the LDL receptor in the current studies remain unknown. While intracellular degradation can be an important regulatory mechanism, proteolysis of membrane receptors within the plasma membrane, as seen in the current studies, has not been commonly observed. The V2 vasopressin receptor has recently been reported to undergo proteolysis by a membrane-associated metalloprotease, a process that is triggered by the binding of vasopressin to the receptor and which can be observed in purified membrane preparations (38). However, the mechanism of the apparent proteolysis of the LDL receptor stimulated by isoproterenol, although also mediated via a G protein-coupled receptor, seems to differ from proteolysis of the V2 receptor, because the ability of isoproterenol to stimulate proteolysis of the LDL receptor was seen only when it was incubated with intact adipocytes, with no effects observed when it was incubated with purified plasma membranes (data not shown).

Similar to our previous observations (10), LDL receptors detected by immunoblotting in subfractions of isolated rat adipose cells are found primarily in intracellular membranes associated with enzyme markers of the Golgi complex, as well as in plasma membranes. In these prior experiments we noted that insulin caused a rapid, 40% reduction in LDL receptors in isolated rat adipocytes, apparently due to an acceleration of the degradation of the LDL receptor (10). However, the diminution of LDL receptors in the experiments with insulin under standard conditions was confined to a reduction in LDL receptors within intracellular membranes, without any changes in LDL receptors found in plasma membranes. In contrast, in the current studies isoproterenol and cyclic AMP decreased intact LDL receptors in plasma membranes without affecting LDL receptors found in intracellular membranes. This difference in the location of the pool of LDL receptors that is depleted might suggest the utilization of unique degradative pathways for LDL receptors in response to different mediators.

There are several reasons for examining the effects of cyclic AMP on the expression of the LDL receptors. First, cyclic AMP has been shown to increase LDL receptors in steroidogenic cells (15, 39, 40). This increase in LDL receptors is mediated primarily at the transcriptional level (16). The effect is not due to a direct action of cyclic AMP on the transcriptional control of the LDL receptor; rather, it appears to be mediated via cyclic AMP-induced changes in cellular sterol metabolism (17). Second, cyclic AMP has been reported to have
variable effects on LDL receptors in cells other than steroidogenic cells. For instance, cyclic AMP, or agents acting via cyclic AMP, has been reported to decrease LDL receptors in cultured fibroblasts, monocytes, hepatocytes, and thyroid cells (8, 11–13), while other studies have reported increased LDL receptor expression in monocytes and hepatocytes (14, 41). Some of the discrepancies observed in the literature are probably due to differences in the time courses and culture conditions utilized. It is of note that the thyrotropin- and cyclic AMP-induced diminution of LDL receptors observed in thyroid cells was apparently mediated by an increase in the degradation of LDL receptors (13). Third, food deprivation is associated with multiple metabolic changes that are mediated primarily through the actions of counterregulatory hormones, such as catecholamines (epinephrine, norepinephrine) and ACTH, whose actions are mediated via cyclic AMP. We have recently observed that depriving rats of food for 2 days results in a 95% reduction of LDL receptors in isolated adipocytes (18). This diminution in LDL receptors was only partially explained by an approximate 50% decrease in LDL receptor mRNA levels and the rate of LDL receptor synthesis, suggesting additional posttranscriptional mechanisms controlling LDL receptor expression. The demonstration that isoproterenol and ACTH decrease the amount of intact LDL receptors in the plasma membranes of isolated adipocytes, as well as the demonstration that isoproterenol rapidly decreases the levels of LDL receptor mRNA in isolated adipose cells, provides a potential mechanism to account for the profound reduction in LDL receptors in adipocytes that occurs with fasting. Furthermore, while the expression of LDL receptors is regulated primarily via transcriptional control mediated by changes in cellular sterols (42), these results highlight the potential importance of posttranscriptional mechanisms in controlling LDL receptor expression under certain conditions.

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