Increased Low Density Lipoprotein Receptor Expression Mediated through the Insulin-Like Growth Factor-I Receptor in Cultured Fibroblasts

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Plasma insulin-like growth factor-I (IGF-I) levels are inversely correlated with apolipoprotein B and low density lipoprotein (LDL) cholesterol in humans. To identify a molecular basis for this observation, the effects of IGF-I on LDL receptor expression in fibroblasts were studied. IGF-I increased LDL receptors in cultured human skin fibroblasts at concentrations greater than 25 ng/ml. However, IGF-I effects were not easily quantitated due to secretion of inhibitory IGF-binding proteins by the cells. To circumvent this difficulty, QAYL, an IGF-I analog that binds to the IGF-I receptor but not to IGF-binding proteins, was used. QAYL increased LDL receptor number 56–72% with half-maximum effect at 0.6 ng/ml. α-IRS, a monoclonal antibody directed toward the IGF-I receptor, blocked this effect. QAYL treatment increased synthesis of LDL receptor protein without increasing LDL receptor mRNA levels or altering protein stability. Both QAYL and IGF-I increased LDL receptors prominently in cells that had been treated with physiological amounts of LDL cholesterol. IGF-I, acting through the IGF-I receptor and modulated by IGF-binding proteins, may contribute to the regulation of LDL metabolism by increasing translation of LDL receptor message. (Molecular Endocrinology 8: 904–909, 1994)

INTRODUCTION

Insulin-like growth factors (IGFs) were originally described as endocrine mediators of the action of GH on cartilage (1), but subsequent work has shown that they are involved in the regulation of numerous cellular processes, binding to receptors in many tissues with important autocrine, paracrine, and endocrine actions (2, 3). In contrast to other growth factors, IGF-I and IGF-II are normally present in plasma at high concentrations (150–700 ng/ml) (3) in association with specific binding proteins, suggesting that they have an endocrine role. The IGFs persist in plasma long after linear growth has ceased.

One potential function for the IGFs in adults is regulation of lipid metabolism. Recently we reported that plasma IGF-I was inversely correlated with apolipoprotein B, the protein component of the low density lipoprotein (LDL) particle, and with LDL cholesterol levels in man (4). To determine whether this relation was due in part to an increase in LDL receptor expression, we studied the effects of IGF-I on LDL metabolism in cultured human skin fibroblasts. While the fibroblast culture system is a good model system for peripheral tissue lipoprotein receptors, the use of IGF-I is complicated by the finding that fibroblasts, like many other cultured cells, actively secrete binding proteins that inhibit IGF-I action (5, 6). To eliminate this difficulty we have used two analogs of IGF-I [Gln3,Ala4,Tyr'5,Leu'6]-IGF-I(QAYL) and [Phe-',Val',Asn2,Gln3,His4,Ser5,His6, Glu7,Tyr8,Leu9]-IGF-I [B-chain mutant (BCM)], which bind normally to the IGF-I receptor but which have very poor affinity for the interfering binding proteins (7). The results show that IGF-I and its receptor-binding analogs increase LDL receptor expression and do so by a mechanism that is independent of the measured levels of LDL receptor mRNA and also of the suppressive effect of exogenous lipoproteins.

RESULTS

IGF-I increased [125I]LDL binding in cultured human skin fibroblasts. When confluent cells were preincubated for
24 h in BSA medium with or without IGF-I, receptor-specific [\(^{125}\)I]LDL binding measured at 4 C was increased to 133.1 ± 8.5% of control by 25 ng/ml IGF-I (six experiments, \(P = 0.01\)) and to 216.7 ± 12.1% of control by 500 ng/ml IGF-I (six experiments, \(P = 0.0002\)). Similar results were obtained with cells assayed for LDL binding, internalization, and degradation at 37 C (data not shown). However, despite these highly significant results, Scatchard plots of [\(^{125}\)I]LDL binding experiments using IGF-I-treated cells were consistently complex and nonlinear. We postulated that variable amounts of IGF-binding proteins secreted into the culture medium by the cells during overnight incubation with IGF-I might have resulted in variable concentrations of free IGF-I over time.

To test this hypothesis, the IGF-I analogs QAYL and BCM, which do not interact significantly with fibroblast-binding proteins, were used. Although these substances have nearly the same affinity for the IGF-I receptor as IGF-I itself, Fig. 1A shows that the analogs (closed symbols) were more than 100 times as potent as IGF-I (open circles) in increasing [\(^{125}\)I]LDL binding. Half-maximum stimulation of [\(^{125}\)I]LDL binding was seen at 0.6 ng/ml QAYL and 0.5 ng/ml BCM, with maximum effects observed at 5 ng/ml. This suggests that IGF-I increases LDL receptor expression with attenuation of the response by binding proteins of cellular origin. Figure 1B gives the time course of LDL receptor increase in response to QAYL. A significant difference between control and QAYL-treated wells first appeared at 4 h (32% increase, \(P = 0.03\)) and continued for at least 48 h.

Figure 2 shows that in intact fibroblasts treated with 5 ng/ml QAYL the principal effect on LDL receptor activity was a 72% increase in LDL-binding capacity (from 7.7 ± 0.7 ng LDL/mg protein to 13.2 ± 0.7 ng/mg, \(P < 0.0001\)). A smaller increase in affinity for LDL was also found as reflected in a 38% reduction in the dissociation constant (Kd) for LDL (from 13.1 ± 2.2 μg/ml to 8.1 ± 0.9 μg/ml, \(P = 0.039\)). The increase in LDL receptor mass was confirmed by immunoblot analysis of triplicate whole cell extracts (data not shown) in which polyclonal anti-LDL receptor antibodies detected 56 ± 10% more receptors per milligram of protein in QAYL-treated cells (\(P = 0.008\)).

The effect of IGF-I and QAYL was apparent under physiological conditions in which LDL was present in the culture medium and LDL receptors were down-regulated. Figure 3 shows that in the presence of LDL, QAYL resulted in a 65 ± 17% (se) increase in specific [\(^{125}\)I]LDL binding (\(P = 0.02\)), and IGF-I caused an 82 ± 27% increase (\(P = 0.01\)). The effect was seen whether...
IGF-I

Fig. 3. Increased LDL Receptor Expression by IGF-I and QAYL in Cells Down-Regulated by LDL

All cells were grown for 4 days in 10% fetal bovine serum and then for 24 h in 50 μg/ml LDL ± 250 ng/ml IGF-I or 50 μg/ml LDL ± 5 ng/ml QAYL as indicated. Either 0.1% BSA (experiment depicted by the right two bars) or 7 mg/ml LPDS (separate experiment, left two bars) was included during the final incubation. The cells were washed twice and incubated with serum-free medium at 37°C for 30 min before placing on ice for measurement of receptor-specific [125I]LDL binding. Results are the mean of six wells with ±SE shown. The effects of QAYL (P = 0.02) and IGF-I (P = 0.01) were both statistically significant.

Fig. 4. Effect of Monoclonal Antibody α-IR3 (Anti-IGF-I Receptor)

Fibroblasts were washed and treated with BSA medium containing 5 ng/ml QAYL, 5 μg/ml α-IR3, 5 μg/ml mouse immunoglobulin G, or no addition. After 24 h the cells were assayed for specific [125I]LDL binding at 4°C. Results are from triplicate wells with ±SE shown. that QAYL increased synthesis of LDL receptor protein as assessed by incorporation of [35S]methionine/cysteine at 1 and 4 h by 87% (P = 0.006). LDL receptor protein stability was unaltered by QAYL as shown in the pulse-chase experiments of Fig. 5C. The half-life of LDL receptor protein was 8.2 ± 0.6 h in control cells and 6.8 ± 0.4 h in QAYL-treated cells (difference not significant).

To examine the generality of LDL receptor induction by growth factors, both fibroblasts and Hep G2 human liver cells were treated for 24 h with a single growth factor in BSA medium and then analyzed for LDL receptors (Table 1). Under these conditions there was no increase in protein per well with any hormone treatment. Both fibroblasts and Hep G2 cells showed significant increases in LDL receptor activity per milligram of protein with IGF-II and transforming growth factor-β1 (TGF/β1), and fibroblasts responded as expected to platelet-derived growth factor-AA (PDGF-AA) treatment. Thus, several growth factors are capable of modifying LDL receptor expression to a similar degree in both fibroblast and liver cells.

DISCUSSION

There are few reports of the effects of IGF-I on cellular LDL metabolism in the literature, and two experimental difficulties seem to be partly responsible. First, LDL receptors often are measured in the presence of LPDS to increase receptor expression to its maximum level. Under these conditions the further addition of IGF-I was without effect. However, a prominent increase in LDL
IGF-I and LDL Receptors

Fig. 5. LDL Receptor mRNA Level, Protein Synthesis, and Protein Turnover after Treatment with 5 ng/ml QAYL (○) or No Hormone (○).

A, LDL receptor mRNA. Cells were washed and incubated in BSA medium with or without QAYL for the indicated time, and LDL receptor mRNA was quantitated in quadruplicate 150-mm plates. Error bars represent SE. B, LDL receptor biosynthesis (see Materials and Methods). Triplicate dishes were incubated 24 h with or without 5 ng/ml QAYL and for the final 1 or 4 h of that time with 150 μCi/ml [35S]methionine/cysteine. Radiolabeled LDL receptors were immunoprecipitated and quantitated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Error bars represent SE. C, LDL receptor stability. Quadruplicate dishes received 45-h pulses of 150 μCi/ml [35S]methionine/cysteine in hormone-free medium and then were washed and chased in duplicate with or without 5 ng/ml QAYL in BSA medium for up to 24 h. At the indicated times after the beginning of the chase period, the radioactivity in LDL receptors was immunoprecipitated and quantitated.

The principal action of IGF-I was to increase LDL receptor number. Figure 2 shows a 72% increase in maximum [125I]LDL binding capacity, and a similar 56% increase in total cell LDL receptor mass was observed in immunoblotting experiments. However, there was also a statistically significant 38% decrease in the dissociation constant for LDL, indicating an increase in affinity of the cells for LDL. The IGF-I receptor seems to mediate these effects, because increased LDL binding was blocked by a monoclonal antibody, α-IR3, which is specific to the IGF-I receptor (Fig. 4).

Although there is a growing body of literature on the clinical effects of IGF-I on LDL metabolism (4, 10, 11), only specialized cells have been studied in tissue culture previously. It has been reported that IGF-I treatment of cultured swine granulosa cells increased LDL receptor number (12); however, because IGF-I stimulated a large increase in progesterone synthesis from cellular cholesterol, it is likely that in this case much of the effect on LDL receptors was due to cholesterol depletion. Recent work has established that murine monocyte-macrophages responded to both GH and IGF-I by increasing LDL degradation (13), consistent with increased LDL receptor activity or more rapid LDL processing. Our data show that the response to IGF-I is principally mediated by increased LDL receptor mass, and that it is not confined to specialized differentiated cells.

The regulation of LDL receptor expression by cholesterol occurs at the level of transcription (14–16). Other hormones such as insulin (17, 18) and PDGF (19) also seem to augment transcription of LDL receptor mRNA. In contrast, LDL receptor mRNA measured with a sensitive solution hybridization assay was unchanged after IGF-I treatment, suggesting a posttranscriptional mechanism of control (Fig. 4). Although not studied as exten-
sively as transcription, translational or posttranslational regulation of LDL receptors has been suggested in liver after fat feeding (15, 16) and in cultured fibroblasts exposed to lipoprotein-deficient serum (20, 21). The current data suggest strongly that IGF-I acts by increasing translation of a constant amount of LDL receptor mRNA (Fig. 5D). The stability of LDL receptor protein was not altered by IGF-I treatment (Fig. 5C).

Translational regulation might, therefore, complement traditional transcriptional control of LDL receptors. For example, IGF-I retains its ability to increase LDL receptors in cells cultured in physiological (and saturating) amounts of LDL (Fig. 3). This suggests that cholesterol and IGF-I may have differing mechanisms of action and implies that IGF-I could up-regulate LDL receptors even when circulating LDL levels are relatively high, as is usually the case in mammals. IGF-I, therefore, while not the principal regulator of LDL receptor expression, nevertheless might modulate LDL receptor expression in a physiologically meaningful way and by a unique mechanism.

**MATERIALS AND METHODS**

**Materials**

Normal human skin fibroblast strain GM5565 obtained from the Human Genetic Mutant Cell Repository (Greenvale, NY) was used for this work. Similar results were observed with two other strains of human deltoid skin fibroblasts initiated by the authors. Recombinant human IGF-I was purchased from R&D Systems (Minneapolis, MN). Human PDGF-AA was purchased from United States Biochemical Corporation (Cleveland, OH). IGF-I and IGF-II were purchased from Bachem Inc. (Torrance, CA). QAYL and BCM were kindly donated by Dr. Margaret Cascieri (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ). QAYL and BCM have the following affinities (relative to IGF-I): 1.1 and 0.5 for the IGF-I receptor; 0.002 and less than 0.0005 for human serum IGF-binding proteins; 3.0 and less than 0.06 for the IGF-II receptor; and 14.0 and 4.0 for the insulin receptor (7). BSA product A-4503 was purchased from Sigma (St. Louis, MO) and found to be free of significant IGF-I, IGF-II, and binding protein activity. The monoclonal anti-IGF-I receptor antibody α-IR3 was a gift of Dr. S. Jacobs (Welcome Institute, Research Triangle Park, NC).

**Methods**

Fibroblasts were plated at 5 × 10⁴ per 16-mm-diameter well in minimal essential medium plus 10% fetal bovine serum containing 1.0 mm sodium pyruvate and 0.1 mm nonessential amino acids on day 0; on days 3 and 5 the medium was replaced. On day 6 the cells were washed twice and then incubated for 24 h with minimal essential medium containing 1.0 mm pyruvate, 0.1 mm nonessential amino acids, and 0.1% BSA (BSA medium) with or without IGF-I or antibodies. [¹²⁵I]LDL binding was then performed as described previously after cooling the cells to 4°C for 40 min using 15 μg/ml [¹²⁵I]LDL with or without 500 μg/ml unlabeled LDL (22, 23). Results reported are specific binding (binding remaining after subtracting residual binding seen in the presence of at least a 10-fold excess of unlabeled LDL) ± SE for triplicate wells. In some assays incubations with radiolabeled LDL were performed at 37°C, and dextran-releasable surface-bound, internalized, and degraded [¹²⁵I]LDLs were calculated. LPDS (23) was found to contain 35 ng/ml IGF-I and was used in some experiments as a control. Scatchard plots of [¹²⁵I]LDL binding with and without QAYL pretreatment of the cells were solved using the computer program SAAM (Resource Facility for Kinetic Analysis, University of Washington, Seattle, WA; 24). Differences ± SE between the parameters with and without QAYL were computed and evaluated with the f test. Total RNA was extracted from 150-mm culture plates using an acid guanidinium procedure (25), and LDL receptor and β-actin mRNAs were measured by solution hybridization and RNase protection using radiolabeled cRNA probes as described previously (26). A standard curve constructed using 10–500 pg synthetic RNA was linear (R² = 0.99), and the SE of replicate plate samples read from it was 10.4%. Human skin fibroblasts grown in 10% LPDS for 48 h lost 50% of as much LDL receptor mRNA by this assay as cells similarly grown but with 50 μg/ml LDL added.

For [³⁵S]methionine incorporation experiments, cells were grown in 60-mm-diameter dishes and incubated in BSA medium with or without 5 ng/ml QAYL for 24 h as described. At 4 h and 1 h before the end of the incubation the cells were washed and resuspended with methionine-cysteine-free BSA medium with or without hormone and containing 150 μg/ml [³⁵S]methionine/cysteine (ICN, Costa Mesa, CA). At the end of the 24-h period the cells were harvested, and labeled LDL receptors were immunoprecipitated by a modification of a previous procedure (27). Receptor extract was preclaved with protein A-agarose beads, and then LDL receptors were immunoprecipitated with polyclonal anti-LDL receptor antibody, and the resulting complexes were adsorbed to protein A-agarose. Labeled LDL receptors were eluted into electrophoresis buffer and separated on 7.5% sodium dodecyl sulfate polyacrylamide gels. LDL receptors were quantitated by densitom-

**Table 1. LDL Receptor Activity in Cultured Cells**

<table>
<thead>
<tr>
<th>Material</th>
<th>Fibroblast</th>
<th>Hep G2</th>
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<tbody>
<tr>
<td></td>
<td>−Hormone</td>
<td>+Hormone</td>
</tr>
<tr>
<td>IGF-I (250 ng/ml)</td>
<td>8.5 ± 0.8</td>
<td>18.3 ± 1.3</td>
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<tr>
<td>OAYL (25 ng/ml)</td>
<td>9.2 ± 0.5</td>
<td>21.0 ± 0.3</td>
</tr>
<tr>
<td>IGF-II (250 ng/ml)</td>
<td>10.3 ± 1.3</td>
<td>18.9 ± 1.0</td>
</tr>
<tr>
<td>PDGF-AA (10 ng/ml)</td>
<td>5.3 ± 1.1</td>
<td>10.4 ± 1.1</td>
</tr>
<tr>
<td>TGFβ1 (10 ng/ml)</td>
<td>4.8 ± 0.9</td>
<td>12.6 ± 1.3</td>
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Cells were treated in with hormones in BSA medium for 24 h at the concentration indicated and then assayed for [¹²⁵I]LDL binding activity at 4°C as described in Materials and Methods. Results are mean ± SE calculated from triplicate wells with or without hormone and excess unlabeled LDL. *P < 0.01 with respect to no hormone treatment.

*P < 0.05 with respect to no hormone treatment.
ety of autoradiograms. To study the stability of newly synthesized LDL receptors, cells were grown for 6 days and pulse labeled with [35S]methionine for 4.5 h, washed, and then chased for 3-24 h in BSA medium with or without 5 ng/ml OAYL. At the end of the variable chase period, cells were harvested for quantitation of immunoprecipitable radiolabeled LDL receptors.

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