Downregulation of High-Density Lipoprotein Receptor in Human Fibroblasts by Insulin and IGF-I

MARK J. OPPENHEIMER, KARIN SUNDQUIST, AND EDWIN L. BIERMAN

High-density lipoprotein (HDL_3) particles bind to a cell surface receptor, thereby promoting the efflux of cholesterol from extrahepatic nonsteroidogenic cells. This receptor appears to be upregulated by increased cell cholesterol content and also may be responsive to the growth state of cells. Because insulin can be mitogenic, the effect of insulin on HDL-receptor function was tested. HDL-receptor activity of cholesterol-loaded fibroblasts was inhibited by insulin treatment. Insulin decreased HDL binding in a log-dose fashion (−25% at 67 nM insulin) in association with increases in [^{3}H]thymidine incorporation into DNA. HDL-mediated cholesterol efflux from cholesterol-loaded cells was diminished by insulin treatment of cells in parallel with decreased HDL binding. Insulin induced reciprocal changes in HDL- and low-density lipoprotein (LDL)-receptor activity. In cells in which these receptors were upregulated by varying cell cholesterol content, insulin increased LDL binding (+88%) and decreased HDL binding (−24%). Insulin-like growth factor I (IGF-I, 100 ng/ml) also significantly decreased HDL binding and HDL-mediated cholesterol efflux to a comparable degree. Pooled human serum similarly induced a reduction in HDL binding to its receptor. These results are consistent with the hypothesis that growth factors in general, and insulin and IGF-I in particular, decrease HDL-receptor activity, possibly to promote retention of cholesterol needed for new membrane synthesis during cell proliferation. Such a mechanism could be partly responsible for accumulation of cholesteryl esters in arterial wall cells during atherogenesis in diabetes mellitus. *Diabetes* 38:117–22, 1989

The pathogenesis of atherosclerosis in diabetes mellitus appears to be multifactorial, with lipid abnormalities playing a potentially important role (1,2). In poorly controlled diabetes, concentrations of plasma low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) are elevated, whereas high-density lipoprotein cholesterol (HDL-chol) levels are decreased. In insulin-dependent (type I) diabetic patients, HDL levels have been shown to be inversely correlated with glycosylated hemoglobin levels (3). Non-insulin-dependent (type II) diabetic patients often have decreased HDL-chol and apolipoprotein AI levels (4). The significant association between low HDL-chol levels and coronary artery disease in nondiabetic subjects has been well established (5).

The underlying mechanism behind the presumed protective effect of HDL against atherosclerosis has not been established. One possibility is that it could involve reverse cholesterol transport, a process by which cholesterol is carried by HDL from peripheral cells to the liver, where it can be further metabolized. The initiating step in this process is postulated to be an interaction of HDL particles with the cell membrane. Evidence has accumulated to support the concept that HDL binding to extrahepatic cells is receptor mediated. HDL_3 particles, free of apolipoproteins B and E, bind to a cell surface site distinct from the LDL receptor (6). High-affinity HDL binding and HDL effects on cell cholesterol efflux both saturate at HDL protein concentrations ~20 μg/ml (6–8). HDL-binding activity is increased with cholesterol loading (upregulation) and decreased when cells are depleted of cholesterol (downregulation). Upregulation is blocked by cycloheximide, suggesting involvement of protein synthesis (8). A membrane-associated HDL-binding protein (110,000 M_r) has been identified in various cell types by ligand blotting (9). The binding activity of this protein is specific for HDL and is upregulated by loading cells with cholesterol, consistent with the presumption that it represents the HDL receptor.

HDL-receptor activity has recently been shown to be re-

From the Department of Medicine, Division of Metabolism, Endocrinology, and Nutrition, University of Washington, Seattle, Washington.

Address correspondence to Edwin L. Bierman, MD, Division of Metabolism, Endocrinology, and Nutrition, RG-26, University of Washington, Seattle, WA 98195.

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sponsive to the growth state of the cell. Platelet-derived growth factor (PDGF) decreases both HDL binding and HDL-mediated cholesterol efflux in cultured fibroblasts (10). In pharmacologic doses, insulin has growth factor properties for mesenchymal cells, at least partly through the insulin-like growth factor I (IGF-I) receptor (11). Hyperinsulinemia is common in type II diabetes mellitus (12) and has been linked with atherosclerosis in both normal (13–15) and diabetic (16–18) populations. One possible reason for this association is that prolonged exposure to moderately elevated insulin levels could decrease HDL-receptor activity in arterial wall cells, thereby promoting cell cholesterol retention in excess of that required for cell proliferation, ultimately contributing to foam cell formation and atherogenesis. In this study, we tested the hypothesis that insulin, acting as a growth factor, will decrease HDL binding and HDL-mediated cell cholesterol efflux.

MATERIALS AND METHODS

Cells. Normal fibroblasts were grown from punch biopsies of skin from normal volunteers. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (growth medium) at 37°C. Cells (2–8 passages) were seeded in 35-mm plastic petri dishes with 2 ml of medium containing 5×10^4 cells. After growing 5–7 days to near confluency, cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) containing 0.2% fatty acid–free bovine serum albumin (Sigma, St. Louis, MO) at pH 7.4 and then incubated with serum-free culture medium containing 0.2% albumin (serum-free medium) for 24 h to partially synchronize cell growth (1st incubation). Cells were then incubated from 18 to 24 h

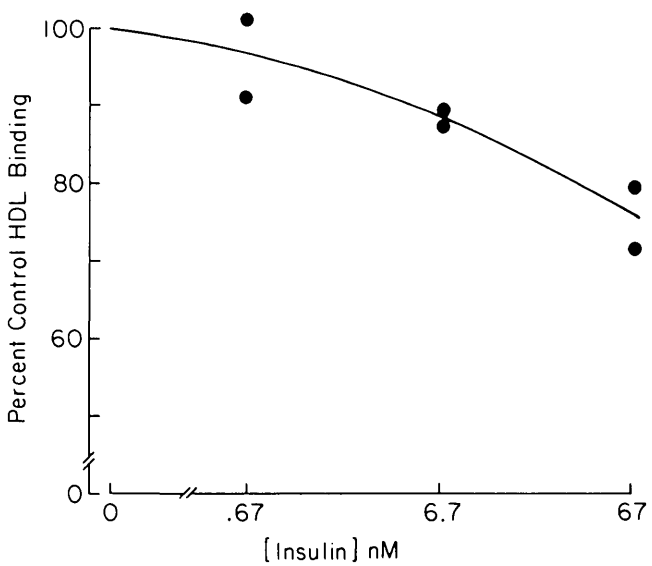

FIG. 1. Dose-dependent effects of insulin on high-density lipoprotein (HDL)-receptor activity. Fibroblasts were preincubated with serum-free medium plus albumin for 24 h before incubation in same medium containing nonlipoprotein cholesterol (50 μg/ml) and indicated concentration of insulin. After 24 h, HDL binding at 37°C was measured as described in MATERIALS AND METHODS. Results of HDL binding are expressed as percentage of control and represent 2 experiments. Each *point* is mean of duplicate experiments. Mean HDL binding with no added insulin (100%) = 84.2 ng HDL protein/mg cell protein. Dose response is significant ($P < .005$ by regression analysis).

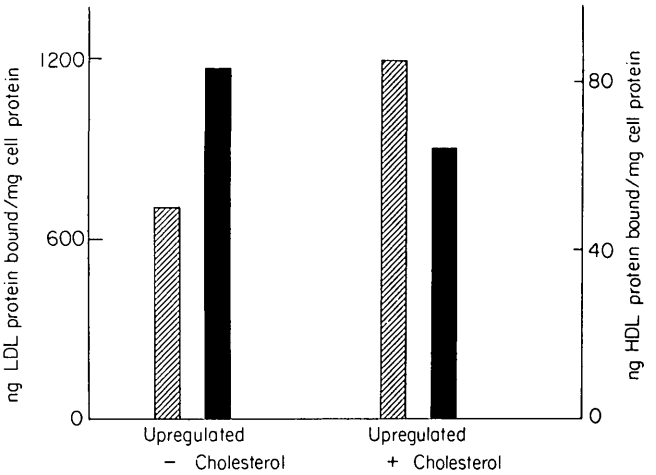

FIG. 2. Effects of insulin on high-density lipoprotein (HDL)- and low-density lipoprotein (LDL)-receptor activity in fibroblasts. Cells were preincubated and treated with insulin as in Fig. 1. HDL- and LDL-receptor activity at 37°C was measured as in MATERIALS AND METHODS. *Bars* indicate mean value of 2 experiments, with each experiment performed in duplicate. *Solid bars*, 67 nM insulin; *hatched bars*, controls. Insulin effects on LDL binding (*left*; $P < .05$) and HDL binding (*right*; $P < .001$) are significant.

in serum-free medium with or without purified porcine insulin (kindly prepared and supplied by C. de Haën, University of Washington; 19), IGF-I (Am-gen, Thousand Oaks, CA) or Imcera (Terre Haute, IN), αIR3 antihuman IGF-I–receptor antibody (kindly supplied by S. Jacobs, Wellcome, London), or 5–10% pooled human serum (second incubation). To load cells with cholesterol, nonlipoprotein cholesterol was added to the medium during either the first or second incubation. Nonlipoprotein cholesterol was dissolved in 100% ethanol (10 mg/ml) before addition to the albumin-containing medium. Because PDGF has been shown to stimulate acyl-cholesterol acyltransferase (ACAT) activity (20), and because HDL binding has been shown to be affected by changes in cholesterol esterification in cells (21), compound 58-035, an inhibitor of ACAT (Sandoz, East Hanover, NJ), was added to incubations (1–2 μg/ml) where indicated to block the possible stimulatory effect of insulin on ACAT activity.

Lipoproteins and PDGF-deficient serum. Lipoproteins were isolated from human serum by refrigerated sequential ultracentrifugation (LDL, d = 1.019–1.063; HDL_3, d = 1.125–1.210) (7,22). Lipoproteins were iodinated by the McFarlane monochloride procedure as modified for lipoproteins (23). Specific activity ranged from 300–500 cpm/ng protein for ^{125}I-labeled HDL_3 and 100–200 cpm/ng protein for ^{125}I-LDL. PDGF-deficient calf serum was prepared by column chromatography with the method of Vogel et al. (24).

Binding of ^{125}I-HDL_3 to cells. To determine cell binding of ^{125}I-HDL_3 at 37°C, cells were rapidly washed twice with PBS-albumin (wash medium) and incubated at 37°C with serum-free medium containing 0.2% fatty acid–free bovine serum albumin and 5 μg/ml ^{125}I-HDL_3 with or without 100 μg/ml unlabeled HDL_3. After 1 h, dishes were chilled on ice, washed rapidly three times with ice-cold wash medium, incubated twice for 10 min with the same medium, and washed twice again rapidly with cold PBS containing no albumin (25). Cells were then digested in 0.1 N NaOH, an aliquot was assayed

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for ^{125}I radioactivity, and another aliquot was assayed for protein content (26). When the assay was performed at 4°C, cells were washed twice at room temperature and chilled to 4°C while bathed with the second wash medium. Cells were then incubated at 4°C with albumin-containing serum-free medium without bicarbonate, 25 mM HEPES (pH 7.4), and ^{125}I-HDL_3 with or without excess unlabeled HDL_3. After 2 h, cells were washed and digested by the same procedure used for the 37°C binding assay (25). The degree of regulation of HDL binding by PDGF (10) and cholesterol (8) was similar when the assays were performed at either temperature. High-affinity specific binding of HDL_3 was calculated as the difference between binding of ^{125}I-HDL_3 in the absence and presence of excess unlabeled HDL_3.

[^{3}H]cholesterol efflux. Subconfluent fibroblasts were washed, incubated with 5% PDGF-deficient serum for 3 days (to attempt to remove the residual influence of PDGF), and then sterol loaded with [^{3}H]cholesterol. Cells were incubated for 36 h with albumin-containing serum-free medium to which the indicated concentration of [^{3}H]cholesterol in ethanol was added, were washed twice with wash buffer, then incubated for 9 h with serum- and cholesterol-free medium (plus albumin) to allow for equilibration of intracellular cholesterol pools and internalization of surface-bound cholesterol. Cells were incubated for 18 h with serum- and cholesterol-free albumin medium with or without insulin.

To assay the rate of efflux of [^{3}H]cholesterol from cells, fibroblasts were washed twice with PBS-albumin and subsequently incubated for 6 h with medium containing 1 mg/ml albumin plus unlabeled HDL_3 (10 μg/ml or the indicated concentrations). Cells were then chilled on ice, the medium was collected and centrifuged to remove any detached cells, and radioactivity was assayed by liquid-scintillation counting. HDL_3-mediated cholesterol efflux is defined as the difference in the amount of tritiated radioactivity appearing in the medium in the presence and absence of HDL_3. Cellular lipids were extracted from washed cells by hexaneisopropanol (27) and separated by thin-layer chromatography (25). Total cellular cholesterol mass was assayed by the cholesterol oxidase method (25,28). More than 90% of the cellular cholesterol was in the free form, reflecting the routine inclusion of the ACAT inhibitor in the incubation media.

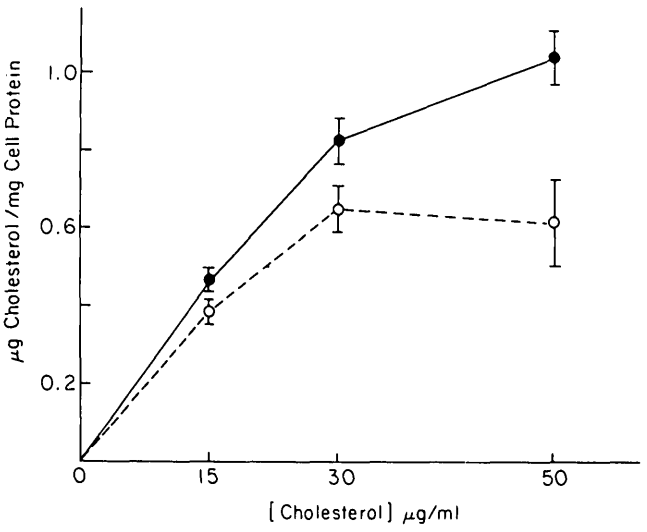

FIG. 3. Effect of insulin on high-density lipoprotein (HDL)-mediated cholesterol efflux. Subconfluent fibroblasts were preincubated with 5% platelet-derived growth factor–deficient serum, washed, then incubated with serum-free medium plus albumin and an acylcholesterol acyltransferase (ACAT) inhibitor with increasing concentrations of [^{3}H]cholesterol for 24 h. Cells were washed and incubated for 9 h with serum-free medium, albumin, and ACAT inhibitor, followed by 18-h treatment with identical medium in presence (○) or absence (●) of insulin (67 nM). After 18 h, cells were washed and then incubated for 6 h at 37°C with or without insulin in serum-free medium, albumin (0.1%), and ACAT inhibitor in presence or absence of HDL_3 (10 μg/ml). HDL-mediated cholesterol efflux was determined as in MATERIALS AND METHODS. Difference in cholesterol (calculated from specific activity of [^{3}H]cholesterol) in medium in presence and absence of HDL_3 is plotted on ordinate. $P < .05$ at cholesterol concentration of 50 μg/ml.

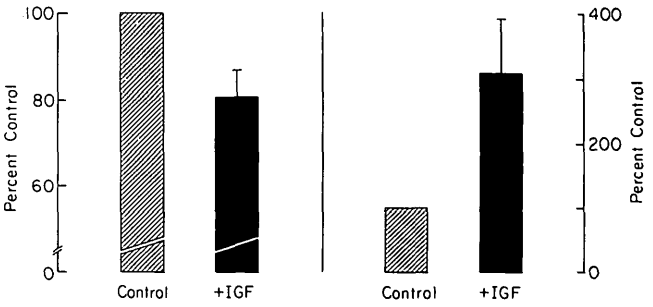

FIG. 4. Effect of insulin-like growth factor (IGF-I) on high-density lipoprotein (HDL)-receptor activity and [^{3}H]thymidine incorporation into DNA. Subconfluent fibroblasts were loaded with cholesterol before or concurrent with incubation with serum-free medium, albumin, and acylcholesterol acyltransferase inhibitor in presence (*solid bars*) or absence (*hatched bars*) of IGF-I (100 ng/ml; 13 nM). HDL binding (*left*) and [^{3}H]thymidine incorporation (*right*) were measured as described in MATERIALS AND METHODS. Results are expressed as percentage of control and represent 9 experiments, with each experiment performed in either duplicate or triplicate. $P < .02$ for HDL binding.

Other methods. [^{3}H]thymidine incorporation was measured at 18 h after the addition of insulin or IGF-I by the method of Pollack and Vogel (29) as previously described (30). [^{3}H]thymidine was added at 2.5 μCi/ml to the incubation medium for 2 h at 37°C. Binding of ^{125}I-LDL to fibroblasts was determined by the same procedure used for the 37°C assay of ^{125}I-HDL_3 binding. Data analysis was performed by Student's *t* test and analysis of variance. The intra-assay coefficient of variation for HDL binding at 4°C was 9.5%.

RESULTS

To assess the effect of insulin on HDL-receptor activity, subconfluent fibroblasts were incubated in medium with or with-

TABLE 1
Effect of insulin-like growth factor I (IGF-I) on specific high-density lipoprotein (HDL) binding and HDL-mediated cholesterol efflux in cultured human skin fibroblasts

Addition	Cell protein (μg/dish)	HDL binding (ng/mg cell protein)	[^{3}H]cholesterol efflux (μg/mg cell protein)
None	96.5	24.8 ± 2.7	46.6 ± 1.9
IGF-I (100 ng/ml)	109.3	19.6 ± 4.8	36.4 ± 1.0

Cells were preincubated for 36 h with 50 μg cholesterol/ml ethanol to upregulate HDL-receptor activity. Incubation time with IGF-I was 18 h. Determination of specific HDL binding at 4°C, and [^{3}H]-cholesterol efflux as described in MATERIALS AND METHODS. Values are means ± SD of triplicate determinations.

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TABLE 2
Effect of insulin-like growth factor I (IGF-I) and IGF-I–receptor antibody on specific high-density lipoprotein (HDL) binding and DNA synthesis in cultured human skin fibroblasts

Addition	Cell protein (μg/dish)	HDL binding (ng/mg cell protein)	[^{3}H]thymidine incorporation into DNA (cpm/dish)
None	105.5	24.2 ± 6.0	14,500
IGF-I (50 ng/ml)	138.9	14.2 ± 3.2 (−41%)	41,750
IGF-I (50 ng/ml) + αIR3 (10 ng/ml)	123.5	25.8 ± 6.0 (+6%)	34,300

Values for specific HDL binding at 4°C are means ±SD for 5 replicate determinations, with percentage of change from control (no additions) in parentheses. αIR3 designates an anti-human IGF-I–receptor antibody. Differences in HDL binding for IGF-I are significant compared with control ($P < .02$) and IGF-I + αIR3 ($P < .01$).

out cholesterol for 24 h in the presence or absence of increasing doses of insulin. Insulin treatment significantly decreased high-affinity HDL binding in a log-dose fashion (Fig. 1) in association with an increase in [^{3}H]thymidine incorporation of 55% at the highest dose of insulin (data not shown). Treatment of cells with insulin had no significant effect on binding of ^{125}I-HDL_3 in the presence of 20- to 100-fold excess unlabeled HDL_3 (low-affinity binding).

Insulin has previously been shown to increase LDL-receptor activity (31,32). When tested in the same experiments, insulin induced reciprocal changes in HDL- and LDL-receptor activity (Fig. 2). In cells depleted of cholesterol, under conditions in which LDL-receptor activity would be upregulated, insulin (67 nM) increased LDL binding (+88%) similarly to observations previously described (31,32). In contrast, insulin treatment of cholesterol-loaded cells with the HDL receptor upregulated decreased HDL binding (−24%, $P < .001$).

To test the effect of insulin on cholesterol efflux, subconfluent fibroblasts were incubated with 5% PDGF-depleted serum for 3 days before treatment with increasing concentrations of [^{3}H]cholesterol for 24 h. After 9 h of incubation with serum-free medium to allow for equilibration of cellular pools, cells were incubated with medium with or without insulin for 18 h. Insulin decreased HDL-mediated cholesterol efflux (Fig. 3), with greatest effects at higher doses of cholesterol pretreatment (−42% at 50 μg cholesterol/ml; $P = .03$). No decrease in total cell-cholesterol mass was detectable.

Some of the insulin-induced stimulation of growth may be mediated through the IGF-I receptor (11). To assess the effect of IGF-I on HDL-receptor activity, subconfluent fibroblasts loaded with cholesterol were incubated with IGF-I (100 ng/ml) for 18 h. In nine experiments (Fig. 4) IGF-I caused a significant decrease in HDL-receptor activity (−19%; $P < .02$) associated with a threefold increase in [^{3}H]thymidine incorporation. In the single experiment in which efflux of [^{3}H]cholesterol in the presence of HDL was determined, IGF-I (100 ng/ml) reduced cholesterol efflux 22%, coupled with a 21% decrease in HDL binding (Table 1).

To test whether the effect of IGF-I or insulin was mediated via the IGF-I receptor, IGF-I (50 ng/ml) (7 nM) or insulin (67 nM) was added to cells with the anti-IGF-I–receptor antibody αIR3 (33). At the concentrations tested, αIR3 partially inhibited the stimulation of cell growth and completely reversed the decrease in HDL binding produced by IGF-I (Table 2). With insulin, the antibody failed to alter [^{3}H]thymidine incorporation into DNA or HDL binding (data not shown).

Because individual growth factors appear to decrease HDL-receptor activity, the effect of pooled human sera, which should contain multiple growth factors, was tested. HDL binding was significantly decreased in a dose-dependent manner ($P < .01$ by analysis of variance) accompanied by increases in [^{3}H]thymidine incorporation into DNA (Table 3).

DISCUSSION

HDL-receptor activity, previously shown to be reduced by exposure to PDGF (10), appears to be comparably responsive to insulin, IGF-I, and serum. These mitogens cause significant reductions in HDL binding associated with increases in cell proliferation. In this study, insulin and IGF-I were shown to decrease HDL-mediated cholesterol efflux as well. Although the insulin concentrations used in this cell culture study were pharmacologic, they provide another model system in which a mitogenic effect is associated with downregulation of HDL receptors.

TABLE 3
Effect of serum on specific high-density lipoprotein (HDL) binding and DNA synthesis in cultured human skin fibroblasts

Pooled human serum in medium (%)	Cell protein (μg/dish)	HDL binding (ng/mg cell protein)	[^{3}H]thymidine incorporation into DNA (cpm/dish)
0	92.6	41.9 ± 5.7	760
5	106.8	28.1 ± 1.2 (−33%)	1650
10	120.0	20.4 ± 0.6 (−51%)	3770

Cells were grown to subconfluence and preincubated for 24 h in serum-free medium containing fatty acid–free albumin. Incubations were performed without or with pooled human serum in the concentrations indicated for 48 h at 37°C. Small dishes (16 mm) were used for thymidine incorporation measurements. Values for specific HDL binding at 4°C are means ± SD for triplicate determinations, with percentage change from control (no added serum) in parentheses.

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It is possible that high insulin levels in vivo in conjunction with other growth factors could contribute to the accelerated atherosclerosis of type II diabetes mellitus. If insulin results in decreased HDL-receptor activity and decreased HDL-mediated cholesterol efflux from arterial wall cells, cholesterol accumulation and foam cell formation could result. This, along with the low circulating-HDL concentrations seen in type II diabetes mellitus (4), could impart additional risk for atherogenesis. Furthermore, insulin also promotes LDL-receptor–mediated uptake (31,32) of cholesterol into cells promoting cholesterol accumulation by yet another mechanism that could be related to hyperinsulinemia.

In contrast, insulin deficiency could promote arterial cell cholesterol deposition by other mechanisms. In uncontrolled type I diabetes, insufficient insulin is associated with elevated LDL-chol levels (34,35), presumably due partly to impaired LDL-receptor–mediated LDL degradation (36). As in LDL-receptor–deficient states caused by gene mutations (37,38), a high LDL-chol level enhances cell cholesteryl ester accumulation, largely by non–receptor-mediated uptake. Low serum HDL-chol levels (particularly the less dense HDL_2 subfraction) in uncontrolled type I diabetes (34,35) adds an additional risk for atherogenesis. In insulin deficiency, low HDL-chol levels could be related to low lipoprotein lipase activity (34) and its associated hypertriglyceridemia (39,40) and may not reflect abnormalities in HDL-receptor interaction.

It is not yet clear how circulating HDL-chol levels relate to changes in HDL_3 binding and HDL-mediated cholesterol efflux measured in cell culture systems. Possibly, HDL_2-chol levels measured in vivo partly reflect the efficiency of HDL_3-mediated cholesterol efflux from extrahepatic cells, but this suggestion remains to be tested. Current regimens of insulin treatment of type I diabetes are associated with variable peripheral insulin levels; hence, it is difficult to predict the level of exposure of arterial wall cells to insulin. Whereas serum HDL-chol levels may be normal (41) or high (42) in treated type I diabetes, higher insulin doses are associated with lower HDL-chol levels (41). Furthermore, in healthy men (43–45) and in subjects with type II diabetes (44,46), higher plasma insulin levels are associated with lower HDL-chol levels, particularly lower HDL_2-chol levels (44,46). Therefore, it seems that low serum HDL_2-chol levels coupled with high arterial insulin levels, both commonly associated with diabetes, might reflect impaired HDL-mediated cholesterol efflux from peripheral cells.

In this regard, PDGF may act in concert with insulin during atherogenesis, since that potent mitogen has similar effects on increasing cholesterol delivery (20,30,47) and decreasing cholesterol efflux (10), thereby also promoting cell cholesterol accumulation. The role of IGF-I in vivo in atherogenesis remains speculative. Results in this study show that IGF-I and insulin both downregulate HDL-receptor activity. Although IGF-I appears to act via binding to its receptor, insulin at the concentration tested (67 nM) does not appear to exert its mitogenic effect via the IGF-I receptor (48). Studies of comparative effects of IGF-I and insulin on fibroblast growth (49) and on interaction with the αIR3 anti-IGF-I–receptor antibody (48) indicate that higher concentrations of insulin than those used in this study are required for insulin to act via the IGF-I receptor.

Thus, it appears that growth factors in general and insulin in particular reduce HDL binding and HDL-mediated cholesterol efflux, and therefore another mechanism for accumulation of cholesteryl ester in arterial wall cells in diabetes mellitus is suggested.

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