Effects of streptozotocin-induced diabetes on low density lipoprotein receptor expression in rat adipose tissue

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Abstract Low density lipoprotein (LDL) receptors are found in most cells, including adipose cells. LDL receptors are primarily regulated by cellular cholesterol content. Insulin and insulin deficiency have been reported to have varying effects on LDL receptors in various tissues. The present study was undertaken to assess the in vivo effects of streptozotocin-induced diabetes on LDL receptor expression and cholesterol content in adipose tissue and liver. Diabetes was induced by a single dose of streptozotocin. After 3 days, some animals were treated with insulin, and all animals were killed 10 days after induction of diabetes. Compared to control rats, 10 days of diabetes caused a decrease in adipose cell size and cellular unesterified cholesterol and cholesteryl esters, and insulin treatment returned these towards normal. No changes were observed in hepatic lipid content with diabetes or insulin treatment. Diabetes was associated with an ~50% reduction in immunoreactive LDL receptors in adipose cells ($P <$ 0.01) that was returned to normal with insulin treatment. The levels of LDL receptor mRNA decreased ~80% ($P < 0.001$) in adipose cells isolated from streptozotocin-induced diabetic rats and returned to normal with insulin treatment. Hepatic LDL receptors and mRNA levels were unaffected by diabetes or insulin treatment. In conclusion, diabetes decreased LDL receptor expression in adipose cells while total cellular cholesterol content also declined.—Swami, S., C. Sztalryd, and F. B. Kraemer. Effects of streptozotocin-induced diabetes on low density lipoprotein receptor expression in rat adipose tissue. *J. Lipid Res.* 1996. **37:** 229–236.

Supplementary key words cellular cholesterol content • adipocyte membrane • mRNA

Low density lipoprotein (LDL) receptors are a member of a family of cell surface receptors that mediate the endocytic uptake of LDL and other apolipoprotein B- and E-containing lipoproteins into cells (1). The expression of LDL receptors is primarily regulated by cellular sterol content, with increases in cellular sterols causing repression and decreases in cellular sterols causing activation of LDL receptor gene transcription via regulation of sterol regulatory element binding proteins (2). In addition to sterols, LDL receptors are regulated by a variety of hormones, including insulin and catecholamines. Insulin has been reported to increase LDL receptor expression in vitro in several cultured cells, such as fibroblasts (3), hepatocytes (4), and monocytes (5). Moreover, insulin appears to increase LDL receptor expression in vivo in man as insulin infusion was observed to accelerate the fractional catabolic rate of LDL (6). In contrast, studies that have examined the effects of insulin deficiency in rats have failed to detect any changes in hepatic LDL receptor expression (7, 8). Catecholamines have generally been reported to decrease LDL receptor expression in a variety of cells, but increases in LDL receptors have also been reported (5, 9–11).

Regulation of LDL receptor expression in adipose cells seems to differ in some aspects from other cells. For instance, we have observed that exposure of isolated rat adipose cells to insulin in vitro causes a rapid decrease in LDL receptor expression while simultaneously increasing glucose transport (12). Furthermore, food deprivation in rats, which is associated with a decline in circulating insulin concentrations but also a concomitant increase in circulating catecholamine concentrations, markedly decreases LDL receptor expression in adipose tissue without affecting hepatic LDL receptor expression (13). The current studies were undertaken to examine the in vivo effects of streptozotocin-induced diabetes on LDL receptor expression in adipose tissue and liver.

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; KRBH, Krebs-Ringer-bicarbonate-HEPES buffer; TES, Tris-EDTA-sucrose buffer; SDS, sodium dodecyl sulfate.

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METHODS

Animals

Male Sprague-Dawley rats weighing 180–220 g were obtained from B and K Inc. (Fremont, CA) and were maintained on commercial rat chow according to the Stanford University guidelines with a 14 h light/10 h dark cycle. Food and water were provided ad libidum. The rats were divided into three groups. Group I consisted of control rats. Group II rats were made diabetic by an injection of streptozotocin (45 mg/kg body weight) dissolved in 0.01 M sodium citrate (pH 4.5) via the tail vein. Group III consisted of diabetic rats that were treated with a variable dose of NPH insulin (5–12 units/day; human insulin isophane suspension) subcutaneously for 7 days. The insulin dose was adjusted daily based on glucose measurements of blood obtained from the tail vein. At the end of 10 days all three groups were killed between 0800–1000 h by decapitation, and plasma obtained was frozen for later analyses. Epididymal fat pads were removed immediately and used for adipose cell isolation. A portion of the fat pad was frozen immediately in liquid nitrogen and used for RNA isolation. Liver was removed and used for crude membrane preparation or frozen in liquid nitrogen and stored at -80°C for RNA isolation.

Adipose cell isolation and membrane preparation

Adipocytes were freshly isolated from epididymal fat pads by collagenase digestion as previously described (13). The fat pads were weighed and minced and collagenase digestion was carried out in Krebs-Ringer-bicarbonate-HEPES (KRBH) buffer (120 mM NaCl, 4 mM KH$_2$PO$_4$, 1 mM MgSO$_4$ 7H$_2$O, 1 mM CaCl$_2$, 10 mM NaHCO$_3$, 27 mM HEPES, pH 7.4) containing 3% BSA and 200 nM adenosine. Cell number was determined by fixing a known aliquot of the cells in a solution of 2% osmium tetroxide in collidine buffer and counting in a Coulter counter. Percent lipid for fat cell depots was determined after Dole extraction (14) and fat cell size (mg triglycerides/10^5 cells) was calculated by dividing the total lipid by adipose cell number. A known aliquot of the cells was kept aside for the separation of unesterified and esterified cholesterol. Total membranes were prepared by homogenizing the remainder of the isolated cells or liver in TES buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 255 mM sucrose and 100 μM leupeptin) at 18°C. The total homogenates were then centrifuged at 100,000 g for 60 min in a 70 Ti rotor to obtain pelleted membranes.

Immunoblot

Total homogenates or membrane proteins were solubilized in 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). After addition of 0.5% SDS and 13% glycerol, electrophoresis was carried out under nonreducing conditions on 8% polyacrylamide gels containing 0.1% SDS (13). After electrophoresis and transfer to nitrocellulose paper, the filters were incubated for 24 h at room temperature with rabbit polyclonal anti-rat LDL receptor antibodies at an approximate final concentration of 10 μg/ml, prepared as described previously (15). This was followed by incubation with 350 ng/ml of ^{125}I-labeled protein A (Amersham Life Science Products, Arlington Heights, IL). The filters were then washed and air dried overnight prior to autoradiography on Kodak XAR film for 5–120 h at -80°C. The relative amounts of LDL receptor contained in each lane was determined by scanning with an LKB ultrascan XL enhanced laser densitometer and gel scan XL software (Pharmacia LKB Biotechnology, Piscataway, NJ) on a NEC computer.

RNA isolation and quantitation

Total cellular RNA was isolated from frozen tissue by chloroform–phenol extraction (16, 17). The RNA pellets obtained were dissolved in sterile water and standard UV absorbance was measured. The RNA was denatured in hybridization buffer (40 mM piperazine-N,N′-bis 2 ethanesulfonic acid, pH 6.4, 400 mM NaCl, 1 mM EDTA, and 80% formamide) and then analyzed by an RNase protection assay (13). The RNase protection assay was developed with a 284 nt fragment of rat LDL receptor cDNA (18) that was subcloned into pBluescript KS II(+). After linearizing with Xho I, the antisense probe was synthesized with T7 RNA polymerase as described by Stratagene to a specific activity of 2.5–5 × 10^8 cpm/μg RNA with [α-^{32}P]CTP. After linearizing with Hga I, an unlabeled sense probe was synthesized with T3 RNA polymerase that yields a 100-bp protected fragment that served as a control for loading in the RNase protection assay. ^{32}P-labeled cRNA probes were then added to tubes containing yeast RNA, formamide-based hybridization buffer, known amounts of sample RNA and sense probes, and then hybridized overnight

TABLE 1. Effects of diabetes on weight and serum lipids

Group (n)	Weight Gain	Glucose	Triglyceride	Cholesterol
	g		mg/dl	
Control (29)	67 ± 3	134 ± 4	76 ± 12	50 ± 4
Diabetic (32)	29 ± 3^a	484 ± 14	228 ± 22^a	64 ± 5
Insulin-treated (8	71 ± 6^c	88 ± 9^{b,c}	88 ± 12^c	61 ± 7

Results are given as mean ± SEM; n, number of animals.
^{a}P < 0.001 compared to control.
^{b}P < 0.05 compared to control.
^{c}P < 0.001 compared to diabetic.

TABLE 2. Effects of diabetes on adipose cell size and number

Group (n)	Total Lipid	Cell Number	Cell Size
	g/g fat pad	10⁵ cells/g fat pad	μg TG/cell
Control (25)	0.201 ± 0.026	5.99 ± 0.68	0.36 ± 0.06
Diabetic (18)	0.065 ± 0.005^a	7.24 ± 0.59	0.15 ± 0.02^a
Insulin-treated (12)	0.173 ± 0.01	7.5 ± 0.54	0.23 ± 0.02

Results are given as mean ± SEM; n, number of animals.
[a]$P < 0.001$ compared to control.

at 45°C. The samples were then digested with RNase T1 and RNase A (60 min at 37°C), followed by proteinase K digestion and phenol–chloroform extraction. The RNase resistant hybrids were recovered by ethanol precipitation and then separated on 6% acrylamide-urea gels. The gels were autoradiographed on Kodak XAR-5 film using intensifying screens at -80°C for 1 day. The autoradiographs were then analyzed by laser densitometry as described above.

Other analyses

Protein was measured with a bicinchoninic acid protein kit (Pierce Chemical Co., Rockford, IL). Plasma was analyzed for glucose, cholesterol, and triglyceride by enzymatic methods using diagnostic kits obtained from Sigma Chemical Co. (St. Louis, MO). Adipose cell and hepatic lipids were extracted with chloroform–methanol 2:1 (19). Cellular cholesteryl esters and unesterified cholesterol from adipose cells were separated by thin-layer chromatography with heptane–ether–acetic acid 80:20:1 using 20 × 20 cm plates precoated with silica gel 150A and quantitated by fluorometry, as described previously (13). Cellular cholesteryl esters and unesterified cholesterol from liver were separated on minicolumns packed with silicic acid–chromosorb W 2:1, eluted with ethyl acetate and benzene, respectively (20), and quantitated using a Sigma cholesterol diagnostic kit.

Statistical analysis

Statistical analyses were performed by analysis of variance and comparisons among groups by Fishers Protected LSD using Super ANOVA software (ABACUS Concepts, Berkeley, CA) on a Macintosh II computer. All results are expressed as mean ± SEM and are representative of three experiments for each group with at least four individual values in each experiment.

RESULTS

Metabolic effects

Rats made diabetic by streptozotocin injection gained ~55% less weight than controls ($P < 0.001$), but insulin treatment normalized the weight gain (**Table 1**). The

reduced weight gain in the diabetic animals was consistent with serum glucose concentrations being 3.6-fold higher in the diabetic group compared to control ($P < 0.001$); insulin treatment corrected the hyperglycemia to values slightly below control. Serum triglyceride concentration was increased 3-fold in the diabetic animals ($P < 0.001$) and was returned to normal with insulin treatment. No differences were observed in total cholesterol concentrations among the experimental groups. The reduced weight gain in the diabetic animals was paralleled by an ~65% decrease in the lipid content of the epididymal fat depot ($P < 0.001$) compared to control that was corrected by insulin treatment (**Table 2**). As no significant changes in adipose cell number in the epididymal fat depot were observed among the experimental groups, the decline in lipid content of the fat depot was due to an ~60% decrease in fat cell size ($P < 0.001$) that was returned to normal with insulin. The reduction in adipose cell size after streptozotocin is similar to that observed by other investigators in previous studies (21).

In addition to a decrease in adipose cell size, i.e., cellular triglyceride, with diabetes, cellular unesterified cholesterol and cholesteryl esters in adipose cells were decreased ~55% with diabetes ($P < 0.005$, **Table 3**). This is consistent with the observation that fat cell size is directly proportional to the amount of cholesterol an adipocyte can accommodate (22). Insulin treatment increased cellular unesterified cholesterol and cholesteryl esters in adipose cells, but did not return the values to normal. In contrast to the alterations in cholesterol content observed in adipose cells, neither diabetes nor insulin treatment was associated with any changes in hepatic unesterified cholesterol or cholesteryl ester content.

Quantity of LDL receptors

In order to determine the amount of LDL receptors, adipose cells were isolated from epididymal fat pads of the experimental groups, and detergent extracts were immunoblotted with rabbit anti-rat LDL receptor anti-

TABLE 3. Tissue cholesterol concentrations in adipose cells and liver

Group	Adipose Cells		Liver	
	Unesterified	Ester	Unesterified	Ester
	μg/10⁶ cell		μg/100 mg wet tissue	
Control (6)	874 ± 126	35 ± 6	214 ± 3	36 ± 3
Diabetic (6	405 ± 59^a	15 ± 2^a	172 ± 22	27 ± 2
Insulin-treated (6)	505 ± 54^b	22 ± 3^c	179 ± 12	32 ± 5

Values are given as mean ± SEM; n, number of animals.
[a]$P < 0.005$ compared to control.
[b]$P < 0.05$ compared to control.
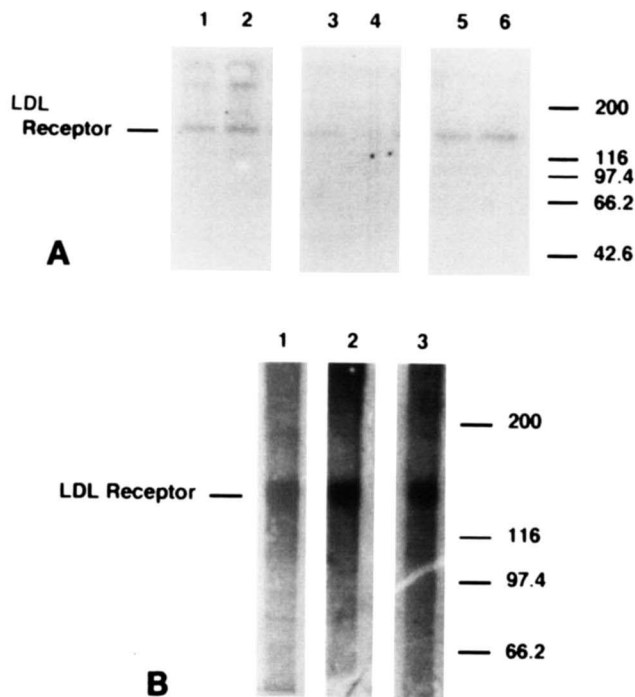[c]$P < 0.01$ compared to control.

Fig. 1. Effects of diabetes on LDL receptor protein expression in adipose cells (A) and liver (B). Adipose cells were isolated from epididymal fat pads from control, diabetic, and insulin-treated diabetic rats. Detergent extracts (50 μg) of total membranes prepared from the isolated adipose cells (A) or liver (B) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with rabbit anti-rat LDL receptor antibodies as described in the Methods. Panel A: lanes 1, 2: control; lanes 3, 4: diabetic; lanes 5, 6: insulin-treated diabetic. Panel B: lane 1, control; lane 2, diabetic; lane 3, insulin-treated diabetic.

bodies (**Fig. 1A**). For comparison, extracts of membranes prepared from livers from the same animals were immunoblotted for LDL receptors (Fig. 1B). The results of densitometric scans of three separate experiments are displayed in **Fig. 2.** Streptozotocin-induced diabetes was associated with an ~50% reduction in LDL receptors

in adipose cells ($P < 0.01$) that was returned to normal with insulin treatment. In contrast, although there was a tendency for immunoreactive LDL receptors in hepatic membranes to increase with diabetes, there were no statistically significant changes with diabetes or insulin treatment. Thus, diabetes is associated with a reduction in LDL receptors in adipose tissue without any changes in hepatic LDL receptors at a time when adipose cholesterol content also declines.

Levels of LDL receptor mRNA

To determine whether alterations in LDL receptor mRNA levels occur with diabetes, steady-state levels of LDL receptor mRNA were assessed in isolated adipose cells and liver from control, diabetic, and insulin-treated groups of rats (**Fig. 3**). When corrected for the amount of sense cRNA added to each hybridization, diabetes was associated with an ~80% decline in LDL receptor mRNA levels in epididymal adipose cells ($P < 0.001$) that was returned to normal by insulin treatment (**Fig. 4**). Thus, the decline in immunoreactive LDL receptors in adipose cells was paralleled by a decline in LDL receptor mRNA. No changes in hepatic LDL receptor mRNA levels were detected in any of the experimental groups, consistent with the lack of changes in immunoreactive hepatic LDL receptors.

DISCUSSION

In the current studies we have shown that diabetes in the rat is associated with a marked decrease in immunoreactive LDL receptors in adipose cells and that this reduction in LDL receptors is corrected by insulin treatment. In contrast, no changes in hepatic LDL receptor expression were observed with diabetes or insu-
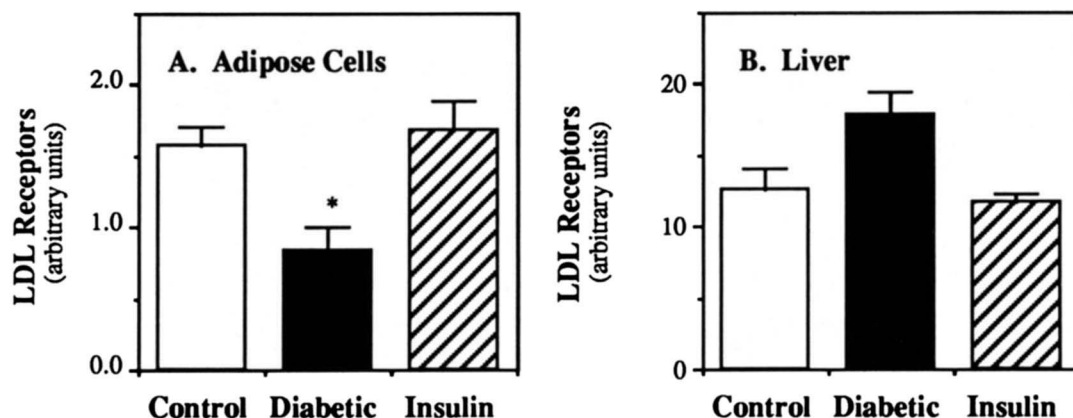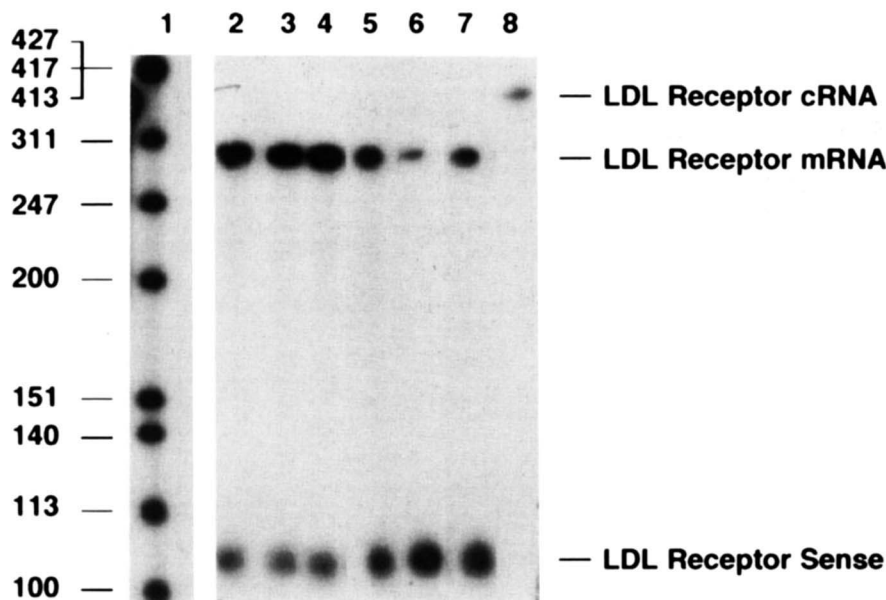

Fig. 2. Densitometric scans of immunoblots of LDL receptors in adipose cells (A) and liver (B). The results are the mean ± SEM of 9 control, 8 diabetic, and 9 insulin-treated diabetic animals and are representative of three separate experiments; *$P < 0.01$ compared to control.

Fig. 3. Effects of diabetes on LDL receptor mRNA levels. Autoradiograph of RNase protection assay of LDL receptor. Lane 1, RNA size ladder; lane 2, RNA from control liver; lane 3, RNA from diabetic liver; lane 4, RNA from insulin-treated liver; lane 5, RNA from control adipose cells; lane 6, RNA from diabetic adipose cells; lane 7, RNA from insulin-treated adipose cells; lane 8, LDL receptor antisense cRNA alone. Total RNA (40 μg) was isolated, hybridized along with 2 ng of LDL receptor sense cRNA with ^{32}P-labeled rat LDL receptor antisense cRNA, treated with RNase, separated on 6% acrylamide-urea gels, and visualized by autoradiography as described in the Methods. The protected LDL receptor RNA fragment is 284 nucleotides and the protected LDL receptor sense cRNA is 100 nucleotides.

lin treatment, consistent with previous reports (7, 8). In diabetic animals, the decrease in LDL receptors in adipose cells appears to be explained by a parallel decrease in LDL receptor mRNA levels, suggesting transcriptional control. However, the decrease in adipose LDL receptors occurred while cellular cholesterol (both unesterified and esterified) decreased; thus, there was an apparent dissociation from the usual inverse relationship between total cellular cholesterol content and LDL receptor expression (1).

Even though with diabetes there was a marked reduction in LDL receptors in isolated adipose cells that was returned to normal by insulin treatment, these alterations in LDL receptor expression occurred concurrently with significant changes in adipose cell size, expressed as triglyceride per cell. These changes in adipose cell triglyceride reflect alterations in adipose cell volume as well as surface area. Because mean fat cell triglyceride is equal to fat cell volume ($\pi D^3/6$, where D is cell diameter) times 0.915 (the density of triolein) (23), the changes in surface area of the adipose cells can be calculated (surface area equals πD^2) from the measurements of triglyceride per cell. Thus, immunoreactive LDL receptors declined by ~50% with diabetes when expressed on the basis of equal amounts of adipose cell protein. In contrast, when immunoreactive LDL receptors in adipose cells are expressed per surface area, there

were no changes in LDL receptors in adipose cells with diabetes (0.136 ± 0.012 versus 0.13 ± 0.026 arbitrary units per $\mu^2 \times 10^3$ in control and diabetes, respectively) while there was a tendency for LDL receptors to increase with insulin treatment (0.196 ± 0.024 arbitrary units per $\mu^2 \times 10^3$); however, this did not reach statistical significance (0.1 < P > 0.05). Although this analysis could lead to a different interpretation of the results, the value of relating LDL receptor expression per fat cell surface area is questionable as we have previously reported that the majority of LDL receptors in adipose cells is localized to intracellular membranes and not to the cell surface (12). In addition, steady state LDL receptor mRNA levels paralleled changes in immunoreactive LDL receptor levels which were expressed on the basis of equal amounts of adipose cell protein. Thus, LDL receptor mRNA levels declined with diabetes and returned to normal with insulin treatment, measurements that would not be expected to be influenced by changes in cell surface area, casting further doubt on the utility of expressing LDL receptor changes in adipose cells on the basis of cell surface area. Therefore, reporting LDL receptor changes in adipose cells on the basis of cell surface area does not appear to be informative, but does highlight the problems inherent to examining changes in specific proteins or RNA in adipose cells during diabetes.
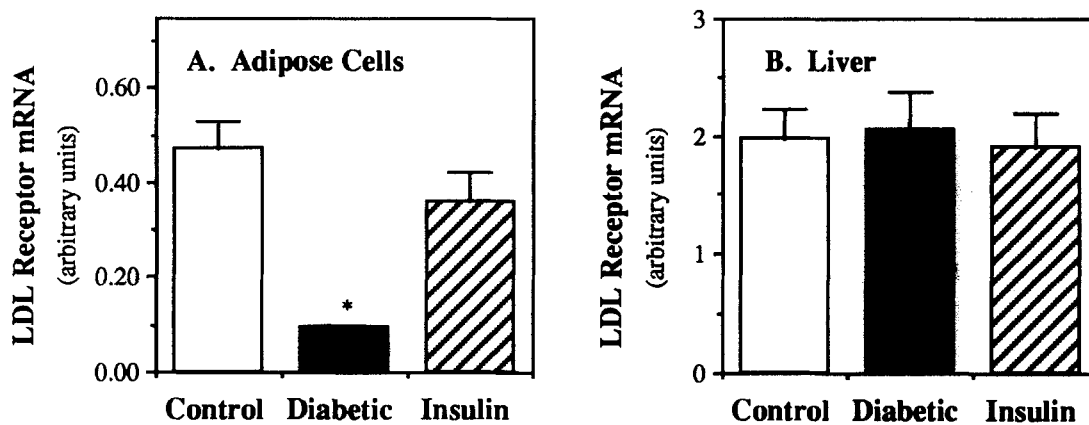
Fig. 4. Densitometric scan of LDL receptor from adipocytes isolated from epididymal fat pads (A) and from liver (B) from control (n = 5), diabetic (5), and insulin-treated diabetic rats (n = 4). Results are the mean ± SEM and are representative of three separate experiments; *,P < 0.001 compared to control.

The reduction in adipose LDL receptor expression with diabetes is similar to that observed in rats during 2 days of food deprivation, where immunoreactive LDL receptors and LDL receptor mRNA levels decreased markedly at a time when adipose cell size and cellular cholesterol content also declined while no changes were observed in hepatic LDL receptor expression (13). Insulin treatment of the streptozotocin-induced diabetic rats returned LDL receptor expression (both immunoreactive protein and mRNA levels) to normal. Taken together, these data might suggest that insulin has a direct stimulatory effect on LDL receptor expression in adipose cells, similar to other cell types where it has been examined (3–5). In contrast, in studies that directly examined the effects of insulin on LDL receptor expression in adipose cells in vitro under conditions free of other possible hormonal influences, we observed a rapid diminution of LDL receptors after exposure of cells to insulin that appeared to be due to an accelerated degradation of LDL receptors (12). It should be noted, however, that in addition to lower insulin levels there are several other hormonal changes that occur in vivo both with streptozotocin-induced diabetes and with food deprivation. In particular, levels of counter-regulatory hormones, such as catecholamines, ACTH, glucagon, etc., are markedly raised under both conditions. Thus, it is possible that the changes in LDL receptor expression in adipose cells observed in the in vivo experiments are not due to direct effects of insulin on LDL receptor expression, but to the effects of counter-regulatory hormones. Indeed this appears to be a likely explanation based on our recent observations that catecholamines and other agents acting through cyclic AMP decrease LDL recep-

tors in isolated adipose cells in vitro by activating a protease that stimulates LDL receptor degradation (24).

Although adipose tissue is generally considered in terms of its role as the major repository of triglyceride in the body, it is also an important site of cholesterol storage (22). Indeed, 15–20% of total body stores of cholesterol are localized to adipose tissue, and even greater percentages are found in obesity (22). Most of the cholesterol contained within adipose tissue is unesterified and, in contrast to most other cells, is non-membrane associated. The unesterified cholesterol is localized to the core of the adipose cell where it is solubilized in the triglyceride droplet. This feature accounts for the finding that the amount of cholesterol stored within a fat cell is proportional to the size of the cell, with larger fat cells, i.e., those containing greater amounts of triglyceride, containing greater amounts of cholesterol (25, 26). In the current studies, diabetes was associated with a >50% reduction in unesterified cholesterol and cholesteryl esters in adipose cells. As total cellular cholesterol declined during diabetes, it is possible that a specific regulatory pool of sterols was expanded due to release of unesterified cholesterol that was solubilized in the triglyceride droplet. The expansion of a specific regulatory pool of sterols would be expected to repress LDL receptor expression through control of sterol regulatory element binding proteins, the primary mechanism regulating LDL receptor expression (2). Thus, the apparent dissociation of the usual inverse relationship between total cellular cholesterol content and LDL receptor expression might be due to some of the unique features of cholesterol homeostasis in adipose cells and, particularly, the way that adipose

cells store cholesterol. Cholesterol homeostasis in adipose cells is dependent on a balance between cholesterol delivery and cholesterol efflux. Alterations in the concentrations or composition of circulating lipoproteins could have influenced cholesterol content in adipose cells. Diabetes caused an increase in serum triglycerides without a change in serum total cholesterol; these changes in serum lipids have been associated with increases in VLDL/IDL cholesterol and decreases in HDL cholesterol levels (27). In addition to these changes in lipoprotein concentrations, alterations in lipoprotein composition occur during diabetes (28) that could affect cellular cholesterol content. However, changes in lipoprotein concentrations and composition do not appear to be important factors affecting LDL receptor expression under the current experimental conditions as these changes would be expected to influence LDL expression in all tissues and hepatic LDL receptor expression was unaltered. Nonetheless, it is possible that there are properties inherent to adipose cells that allow LDL receptors to respond differently to changes in circulating lipoproteins than hepatic LDL receptors. Finally, it is possible that alterations in the expression of other lipoprotein receptors, e.g., the low density lipoprotein receptor related protein/α_2 macroglobulin receptor (29), the VLDL receptor (30), the scavenger receptor B-I (31), or the high density lipoprotein receptor (32), found in adipose cells could have influenced cellular cholesterol content in adipose tissue during diabetes, but these possibilities were not explored in the current studies.

In summary, streptozotocin-induced diabetes in the rat causes hyperglycemia and hypertriglyceridemia with a reduction in adipose cell size, i.e., adipose cell triglyceride, and in adipose cell content of unesterified and esterified cholesterol. Accompanying these changes in adipose cell lipid content, there is a marked decrease in the expression of immunoreactive LDL receptors that appears to be due to a parallel reduction in steady-state LDL receptor mRNA levels. In contrast, diabetes has no effects on hepatic LDL receptor expression or hepatic cholesterol content. Insulin treatment returns LDL receptor expression in adipose cells to normal. These results demonstrate how LDL receptors in adipose cells respond differently than other tissues during diabetes, with LDL receptors decreasing along with total cellular cholesterol content in adipose cells. ∎

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