Lipoprotein-induced Insulin Resistance in Aortic Endothelium

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SUMMARY

An in vitro model system employing cultured, adult, bovine aortic endothelial cells was used to study the mechanism of insulin stimulation of aminoisobutyric acid (AIB) uptake and the effects of low-density lipoprotein (LDL), malondialdehyde-altered LDL (MDA-LDL), and B-migrating very-low-density lipoprotein (B-VLDL) on this process. The insulin response was maximal after treatment with insulin for 2 h (at a concentration of 5×10^{-8} M). Insulin increased the V_{max} but not the K_M of the uptake response. Increasing the cell cholesterol content by a 3-day incubation with malondialdehyde-altered low-density lipoprotein or B-very-low-density lipoprotein, but not low-density lipoprotein, resulted in resistance to the action of insulin. This resistance was not due to a decreased number of insulin receptors or to a decreased receptor affinity. Additionally, the resistance was not abolished by increasing the time of insulin exposure or the concentration of insulin to which the cells were exposed. These findings suggest a postreceptor defect either at the membrane level or intracellularly. DIABETES 1984; 33:1039–44.

Accelerated large vessel atherosclerotic disease is the most common complication of diabetes mellitus today.[1] In most individuals at risk, there is altered lipoprotein metabolism and, especially in type II diabetic subjects, there is insulin resistance.[2,3] The relationship between altered lipoproteins and diabetic atherosclerosis is not understood. Several theories concerning the pathogenesis of the atherosclerotic lesion in diabetes have been advanced;[4,5] all have postulated an initial alteration in the endothelial cells lining large vessels. In this study, we examine the hypotheses that (1) the metabolism of aortic endothelial cells is regulated by insulin, and (2) exposure of cells to certain lipoproteins essentially alters the cellular insulin response. Since insulin responsiveness may be necessary for the endothelial cell to perform its many roles in maintenance of normal vessel wall homeostasis, alterations in the regulatory effects of insulin could cause alterations in the vessel wall, making it more susceptible to atherosclerosis.

We have examined the binding of [^{125}I]-insulin to cultured bovine aortic endothelium and the effect of insulin on transport of amino acids into these cells. The effect of various lipoproteins on the ability of insulin to bind and induce transport was also studied. The lipoproteins examined were ones that may be altered in individuals at high risk. Low-density lipoprotein (LDL) has been shown to be elevated in some studies of diabetic subjects and it possesses a higher cholesterol/protein ratio in diabetic animal models and humans than in nondiabetic subjects.[6,7] Malondialdehyde-altered LDL is a chemically modified LDL that may be made in vivo in diabetic subjects from MDA released from their more labile platelets.[8] Finally, hypercholesterolemic very-low-density lipoprotein, B-VLDL, represents an extreme case of hyperlipidemic VLDL found in high amounts in type II diabetic subjects.[9] In a separate study, we have shown that aortic endothelium possesses separate receptors for each of these three lipoproteins.[10] The present studies have been performed employing primary and secondary cultures of bovine aortic endothelium.

MATERIALS AND METHODS

Materials. Porcine crystalline insulin was generously supplied by Dr. Mary Root, Eli Lilly and Company (Indianapolis, Indiana). [^{125}I]-iodoinsulin was prepared by the chloramine-T method as modified by Freychet et al.[11] and had a specific activity of 100–150 μCi/μg. [^{14}C]-AIB, 19–25 mCi/mmol, was obtained from New England Nuclear (Boston, Massachusetts); Waymouth's medium was from Gibco (Grand Island, New York, cat. no. 320–1220); and fetal bovine serum was from Hyclone, Logan, Utah.

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Cell culture. Bovine endothelial cells were obtained from segments of adult bovine thoracic aortas and were isolated using a 0.5% collagenase solution as previously described.[12] Cells were maintained at 37°C in a 5% CO_2 atmosphere in Waymouth's medium containing 20 U mycostatin, 100 U penicillin, and 100 μg streptomycin per milliliter (medium A) supplemented with 8% fetal bovine serum.

Primary and first passage cultures of endothelial cells at a density of 1–5 × 10^5/cm^2 were used in these studies. These cells displayed the morphologic and growth characteristics of endothelial cells forming a monolayer of flattened polygonal cells. At a density of 4–5 × 10^5 cells/cm^2, >95% of the cells exhibited strict contact inhibition as verified by thymidine incorporation studies with autoradiography.[13] Their endothelial identity was shown by >95% of the cells exhibiting positive immunofluorescence for the presence of factor VIII antigen.[14] Cultures at low cell density (1–2 × 10^5 cells/cm^2), employed in all studies, unless specified, appeared confluent, but were not contact inhibited as assessed by thymidine incorporation (data not shown).

Lipoproteins. Human VLDL (d < 1.006 g/ml) and LDL from normal fasted subjects (d = 1.019–1.063 g/ml) were isolated by ultracentrifugation as previously described.[15] Rabbit B-VLDL and MDA-LDL were prepared as described.[8,16] For lipoprotein exposure, cells were incubated for 3 days with 100 μg/ml of lipoprotein. This is a condition we have previously shown to cause increased cholesterol content of cells incubated with B-VLDL or MDA-LDL.[10]

Amino acid uptake. Cells were plated at 4 × 10^4/cm^2 (unless specified) in 24-well dishes (Costar 3224) in medium A and incubated for 4 days. At this time, the cells were placed in Waymouth's medium containing 1% serum for 18 h, after which insulin was added to test wells without changing the medium. Two hours later, the cells were washed three times with Dulbecco's phosphate-buffered saline (PBS), and then Earles medium, pH 7.4, containing 0.2 (Figure 1) or 0.4 μCi/ml [^{14}C]-AIB (other figures and tables) with or without unlabeled AIB was added to each well. Wells that had contained insulin also received fresh insulin. All wells were incubated at 37°C. At the times indicated, the cells were washed 5 times with PBS and solubilized in 0.8 ml of 0.1 N NaOH. An aliquot (0.3 ml) was counted in Aquasol and another aliquot (0.3 ml) was used to determine protein content by the Lowry method.[17]

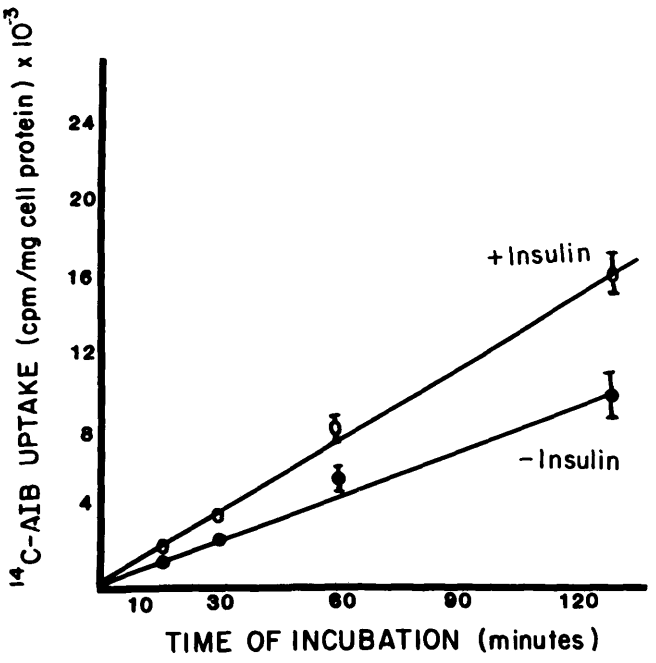

FIGURE 1. Time course of AIB uptake. Values are given as AIB uptake in (cpm/mg protein) × 10^{-3} ± SEM for triplicate measurement for the times shown on the x-axis. Where standard errors are not shown, they are within the point.

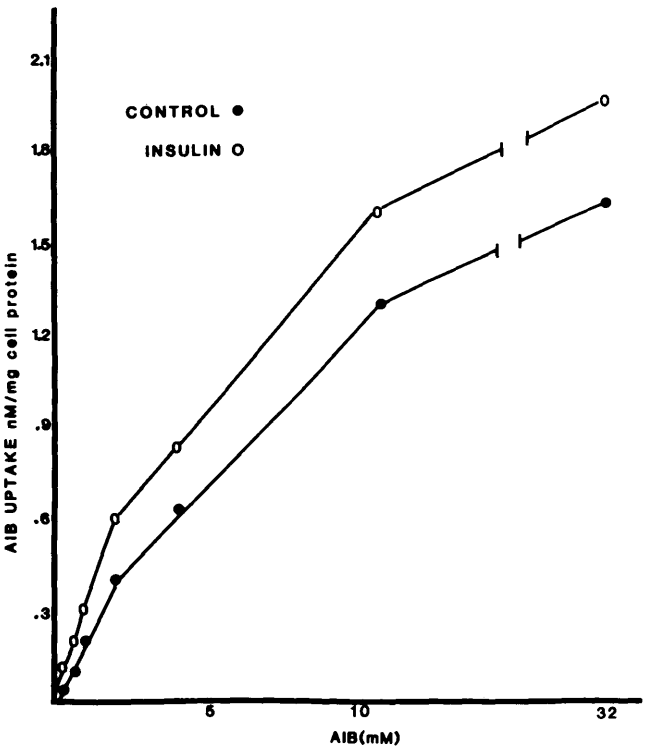

FIGURE 2. The rate of AIB uptake as a function of AIB concentration. Triplicate measurements were employed for each AIB concentration. Uptake rates are reported as nM/mg protein/min of incubation. Standard errors were approximately 15% of the mean.

Cell viability and cell number. The method discussed by Phillips was used to determine cell viability.[18] Briefly, cells were washed 3 times with PBS then removed from the dish with PBS containing 0.12% trypsin and 0.0025 M EDTA; after 3 min, 0.1% trypsin inhibitor was added and the cells were centrifuged at 500 × *g* for 3 min. The cells were resuspended in PBS containing 0.05% trypan blue, and the percent of cells containing dye was determined in a hemacytometer after 3 min in the dye solution.

Insulin binding. Cells were incubated with 0.2 μCi [^{125}I]-insulin (100 μCi/μg) in the presence of 0, 1, 3, 6, 10, 30, 100, or 300 ng/ml unlabeled insulin in PBS plus 0.1% BSA, pH 7.4, for 2 h at 15°C, sufficient time to reach equilibrium. At the end of the incubation, each plate was washed rapidly 3 times with 1 ml ice-cold PBS. The cells were then dissolved in 0.7 ml of 1N NaOH, scraped into counting tubes, and counted in a Nuclear Chicago (Chicago, Illinois) gamma counter. An aliquot was saved to determine the protein by the method of Lowry. Nonspecific binding, binding in the

presence of 100 μg/ml unlabeled insulin, was subtracted from each sample. Insulin degradation was <5% by TCA precipitation of the supernatants.

RESULTS

Kinetics of AIB uptake. The uptake of AIB was linear for 2 h in the presence or absence of 10^{-7} M insulin (Figure 1). The amount of maximal stimulation of AIB uptake by insulin varied between experiments from 130 to 167% of the control value of 9 × 10^3 cpm/mg protein/2 h. The rate of AIB uptake was linear to approximately 4 mM in the presence or absence of insulin (Figure 2). The rate of AIB transport continued to increase at substrate concentrations greater than 4 mM faster than one would have predicted from Michaelis-Menton kinetics. This result is undoubtedly due to the presence of nonsaturable and nonspecific transport systems. The Lineweaver-Burke plots of the 2-h rates of AIB transport between 1 and 32 mM were concave down (data not shown), again indicating the presence of additional transport systems. However, Lineweaver-Burke plots of transport rates between 0.1 and 2 mM were linear, indicating that the nonsaturable component of transport did not have a significant contribution in this substrate range (Figure 3). The insulin-treated cells exhibited a higher V_{max} (2.3 nmol/mg cell protein/min for insulin-treated versus 1.4 for non-insulin-treated), while the K_M was similar in both cases, approximately 3.4 mM.

Effect of lipoprotein uptake on insulin stimulation. The ability of insulin to stimulate AIB uptake in cells incubated for 3 days with the 3 different lipoproteins was examined. We had previously shown that incubation with B-VLDL or MDA-LDL increased the cell cholesterol content by 160–180%, whereas the unmodified LDL did not cause a significant elevation.[10] Cells incubated for 3 days with MDA-LDL or B-VLDL showed no significant insulin stimulation (Table 1). Incubation with lipoproteins did not alter the basal uptake rate. The effect of increasing concentrations of insulin on the AIB uptake response in cells with and without incubation with MDA-LDL was examined (Figure 4). The insulin response was maximal in non-lipoprotein-treated cells at approximately 5 × 10^{-8} M. Increasing the insulin concentration to 10^{-5} M in the medium did not further stimulate AIB uptake in cells preincubated with MDA-LDL. The effect of an increased time of insulin exposure in cells with and without preincubation with MDA-LDL or B-VLDL was also examined (Table 2). The optimum exposure time for cells that were not preincubated was 2 h. Even 12 h of insulin exposure did not increase the insulin response in cells pretreated with lipoprotein.

The insulin resistance was not explained by toxic effects

TABLE 1
The ability of insulin to stimulate AIB uptake in cells preincubated for 3 days with lipoproteins

Lipoprotein added	AIB uptake (cpm/mg protein) × 10^{-3}	
	− Insulin	+ Insulin
None	14.5 ± 0.5	23.7 ± 0.7*
LDL	13.7 ± 0.6	22.8 ± 1.0*
MDA-LDL	15.0 ± 0.5	16.8 ± 0.9
B-VLDL	13.9 ± 0.6	14.6 ± 0.7

Values are given as AIB uptake in (cpm/mg protein) × 10^{-3} (for the 2-h period of AIB incubation). The values given represent the mean of 6 replicates ± SEM.
*$P < 0.005$ for insulin versus non-insulin.

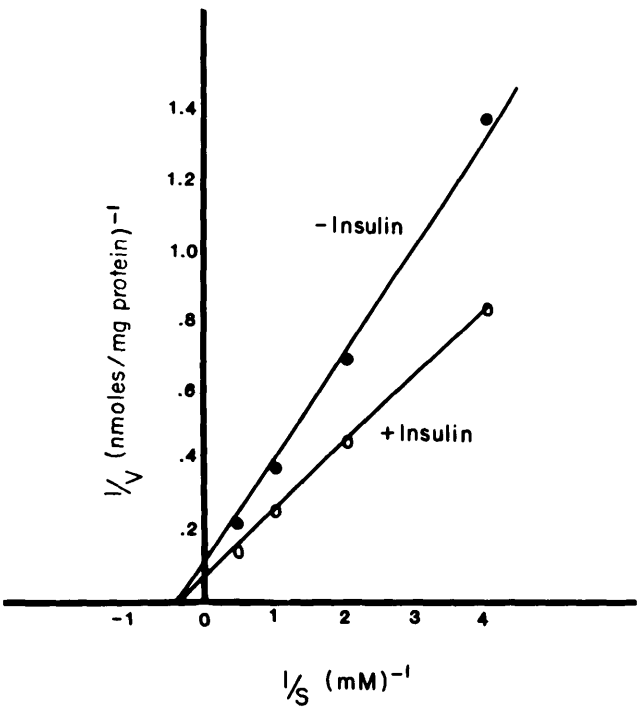

FIGURE 3. Lineweaver-Burke plot of the data in Figure 2 between 0.25 and 2 mM.

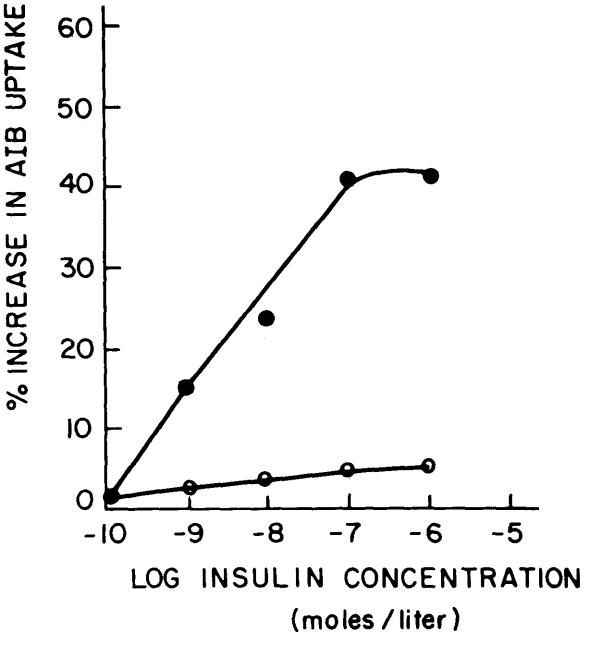

FIGURE 4. Ability of increasing concentrations of insulin to stimulate AIB uptake in untreated cells (●——●) and cells preincubated with MDA-LDL (○——○) for 3 days. Cells were incubated for 2 h with AIB. Data are given as percent stimulation by insulin above the unstimulated value for AIB uptake at 2 h. Values were run in quadruplicate and SEM averaged 12% of the mean.

TABLE 2
Effect of increasing the time of insulin exposure on cells pretreated with B-VLDL and MDA-LDL

Lipoprotein	AIB uptake (cpm/mg protein) × 10^{-3}		
	− Insulin	+ Insulin (2 h)	+ Insulin (12 h)
None	19.7 ± 1.1	29.7 ± 1.4*	28.6 ± 1.2*
MDA-LDL	18.8 ± 0.7	17.4 ± 1.0	18.8 ± 0.7
B-VLDL	19.0 ± 0.6	17.5 ± 0.9	19.6 ± 0.8

Cells were incubated with 10^{-7} M insulin for 2 or 12 h. After this time, all cells were incubated for 2 h with [^{14}C]-AIB. Values are given as AIB uptake in (cpm/mg protein) × 10^{-3} for the 2-h AIB incubation ± SEM of 4 replicates.
*$P < 0.001$ compared with non-insulin. The lipoprotein-pretreated cells showed no stimulation by insulin at 2 or 12 h.

of lipoproteins on the cells (Table 3). Cells incubated with lipoprotein for 3 days completely excluded trypan blue. The number of cells/well and protein/well was the same in cells incubated with and without lipoprotein. A more stringent test, replating lipoprotein-treated cells into normal medium and subsequent regrowth, was similar in untreated and lipoprotein-treated cells.

Insulin binding. Insulin binding was compared in untreated cells and cells preincubated for 3 days with MDA-LDL or B-VLDL. Typical Scatchard plots from these studies are shown in Figure 5. There was no significant difference in the affinity or capacity of the receptors in the untreated or lipoprotein-treated cells. The insulin binding data were analyzed for the equilibrium binding associative constants and the receptor site number assuming a two-site model according to Feldman,[19] as previously described.[20] Typical values for total capacity (R_0) ranged from 38,000 to 60,000 sites per cell depending on the particular cell isolate, and the equilibrium affinity constants (K_A) ranged from 3.9 to 14.0 nM^{-1} for the high-affinity and from 0.028 to 0.062 nM^{-1} for the low-affinity constants. Within a given cell isolate, the values were very consistent, within ±10% on all the R_0 and K_A values, independent of the lipoprotein preincubation.

DISCUSSION

Endothelial cells are important modulators of vascular function, producing substances that regulate smooth muscle cell growth[21] and contraction[22] as well as substances that modulate clot formation.[14,23-25] The amount of these products, where they have been examined, is regulated by the functional state of the endothelium, with observed differences between dividing and nondividing cells.[22,24] In addition, the endothelium serves as a barrier to the movement of molecules from the blood to the underlying vascular wall.[26] For performance of this function, the intactness of the monolayer must be maintained. Atherosclerotic plaques are known to form in vessel regions where endothelial cells are constantly being replaced (probably because of flow stress) in contrast to other vessel areas where endothelial cells have a very long half-life.[27] It has been suggested that the extent of atherogenesis inversely correlates with the efficiency of endothelial cell replacement.[27] Insulin and insulin-like growth factors are known regulators of cell division,[28,29] and, in other cell types, are regulators of the uptake of metabolites prerequisite to cell division[30,31] as is seen in bovine aortic endothelial cells in this study. Thus, the regulation of endothelial cell metabolism is a key control point in vascular homeostasis, and insulin can control the relevant metabolic processes.

Previous studies have shown that endothelial cells of both large and small vessels possess insulin receptors,[20,32,33] and, in small vessels, insulin increases the rate of glycogen formation from glucose and the rate of cell division. We have shown that insulin is able to stimulate the uptake of amino acids in large-vessel endothelium by as much as 50% above the basal rate. We have also found that insulin stimulates amino acid uptake to an even greater extent in small-vessel endothelium.[34] Kinetically, insulin increased the amino acid uptake by increasing the V_{max} but not the K_M. This implies that the number of AIB transport sites, but not their affinity, is increased by insulin. A similar mechanism of insulin regulation of amino acid transport has been observed in isolated liver cells[30] and in liver cells in culture.[31] Insulin has also been shown to regulate glucose transport by increasing the number of transporter molecules in adipose cells.[35,36]

The regulatory effect of insulin on AIB transport was altered by incubating the endothelium with B-VLDL or MDA-LDL. These lipoproteins increased the total cell cholesterol content and caused resistance of the cells to the action of insulin. The resistance appears to be due to a postreceptor defect, since the binding of insulin to cells treated with these lipo-

TABLE 3
Evaluation of lipoprotein toxicity

	Untreated	LDL	B-VLDL	MDA-LDL
Trypan exclusion (%)	100	100	100	100
Protein mg/well	61 ± 5	60 ± 5	63 ± 4	66 ± 5
Cell no. 10^{-5}/well	3.1 ± 0.2	3.0 ± 0.2	3.3 ± 0.2	3.3 ± 0.3
Passage 1 cell no. 10^{-5}/well	2.1 ± 0.2	2.2 ± 0.1	2.6 ± 0.2	2.6 ± 0.3

Cells were incubated for 3 days with LDL, B-VLDL, and MDA-LDL. Cell viability data were then analyzed in terms of trypan exclusion, protein/well, and cell no./well after the 3-day period. In addition, the cell number 4 days after passage of treated and untreated cells is given. All values represent 6 replicate wells ± SEM.

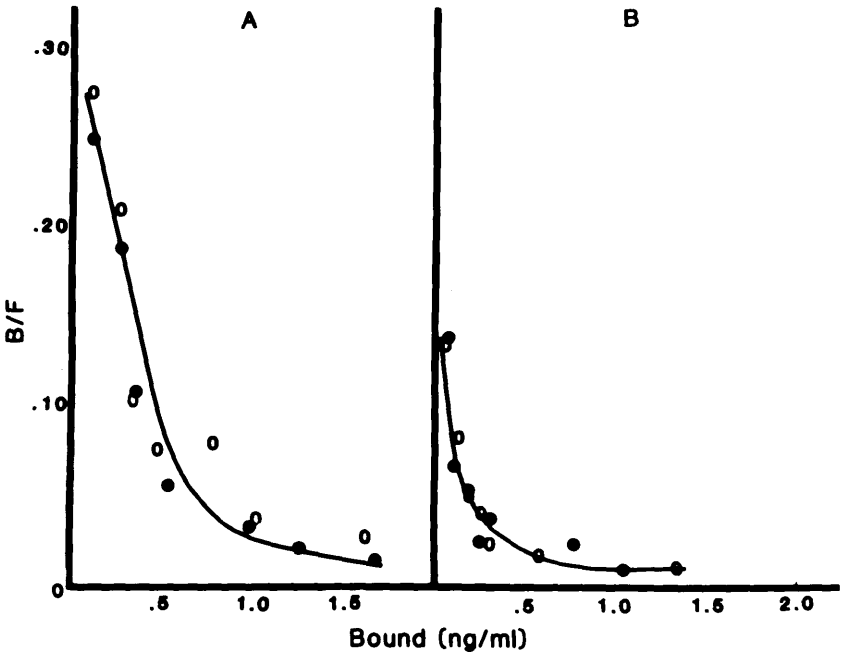

FIGURE 5. Effect of MDA-LDL on insulin binding to endothelium. Scatchard plots of the binding activity are shown. Panel A shows the results for untreated (○——○) and B-VLDL-treated (●——●) cells. Panel B is a separate cell isolate showing data for untreated (○——○) and MDA-LDL-treated (●——●) cells. There was no significant difference in the control and MDA-LDL-treated cells.

proteins was not altered (Figure 5). The fact that LDL did not cause either increased cholesterol content or insulin resistance suggests a connection between cell cholesterol level and insulin resistance, rather than a direct effect of the lipoprotein per se on insulin action.

Elevation of total cell cholesterol content is reflected in increased cell membrane cholesterol content in a variety of cell types.[37] The parallel increase of membrane and total cell cholesterol content presumably occurs in the MDA-LDL- and B-VLDL-treated endothelial cells that have been shown to have an elevated total cell cholesterol content.[10] Increased cell membrane cholesterol is known to alter membrane fluidity,[38] and a number of membrane-related functions, including Na-K exchange,[38,39] in which altered function has been attributed to decreased molecular movement within the cell membrane. At least two steps in the induction of AIB transport by insulin may involve conformation in the cell membrane: (1) Changes in molecular conformation have been implicated in insulin action via its receptor.[40-42] (2) In addition, one interpretation of our present studies and previous work is that insulin increases AIB uptake by the recruitment of transporter molecules into the membrane (Figure 3).[30,31]

Insulin resistance is a common observation in type II diabetes and has also been seen in some type I diabetic subjects.[43] In some of these individuals, resistance is correlated with decreased receptor numbers and can be corrected by increasing the blood insulin levels. In other individuals, increasing the insulin level does not correct the resistance.[43] Although these latter individuals exhibit a lower number of insulin receptors, receptor number alone does not appear to explain the insulin resistance observed.[43] Thus, part of the resistance is due to a postreceptor defect. Within the same cell type, the large adipocyte, some metabolic processes exhibit a postreceptor defect whereas others do not.[43-45] In the metabolic processes in which postreceptor defects have been carefully examined, the authors concluded that defects in the metabolic pathway, rather than the coupling of the insulin receptor to the pathway, were involved.[37-39] By analogy, the resistance to the insulin effect on AIB transport in endothelium may be caused by defects in the production or insertion of transporter molecules. This may be due to altered cell membrane cholesterol content or to other as yet unknown causes.

Obese, type II diabetic subjects frequently exhibit altered lipoprotein levels and composition. For example, LDL and VLDL levels are elevated and protein/triglyceride/cholesterol ratios are altered;[6,7,9] glycosylated LDL is also present but has not been studied in our system.[46] Steiner et al. have shown that nondiabetic patients with endogenous hypertriglyceridemia demonstrate insulin resistance to stimulation of glucose utilization.[47] Results of the present study suggest that altered lipoproteins, although not causing the initial diabetic insult, may contribute to subsequent insulin resistance and perhaps play a role in the accelerated atherosclerotic disease that accompanies diabetes. There are still many steps necessary to prove that altered lipoproteins in human diabetic subjects contribute to insulin resistance. However, these studies would suggest that control of lipoprotein levels is important in controlling insulin resistance.

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