Modulation of a Glycoprotein Recognition System on Rat Hepatic Endothelial Cells by Glucose and Diabetes Mellitus

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ABSTRACT The cellular location and carbohydrate specificities of a glycoprotein recognition system on rat hepatic sinusoidal cells have been determined. Purified preparations of endothelial, Kupffer, and parenchymal cells were prepared by collagenase liver perfusion, centrifugation on Percoll gradients, and centrifugal elutriation. ^{125}I-labeled agalactoorosomucoid, an *N*-acetylglucosamine-terminated glycoprotein, was selectively taken up in vitro by endothelial cells. Uptake was shown to be protein dependent, calcium ion dependent, and saturable, and could be described by Michaelis-Menten kinetics (apparent K_m 0.29 μM; apparent maximum velocity 4.8 pmol/h per 5×10^6 cells). Uptake was inhibited not only by *N*-acetylglucosamine, mannose, and mannan but also by glucose, fructose, and a glucose-albumin conjugate. Inhibition by glucose was competitive over a wide range of concentrations and was almost 100% at a glucose concentration of 56 mM. Fasting and the induction of diabetes mellitus prior to isolation of cells was associated with 60% reductions in the recovery of endothelial cells. Uptake by cells isolated from fasted rats was enhanced (apparent maximum velocity 14.3 pmol/h per 5×10^6 cells without change in the apparent K_m). These observations suggest that fasting is associated with a marked increase in the mean number of glycoprotein receptors per endothelial cell isolated from normal rats. This effect of fasting could be due to upregulation of glycoprotein receptors on endothelial cells or to the selective isolation of a subpopulation of endothelial cells from fasted animals that bears more glycoprotein receptors per cell than does another subpopulation of these cells. In addition, in vivo studies of the fate of intravenously administered ^{125}I-agalactoorosomucoid indicated that its rate of disappearance from plasma, hepatic accumulation, and catabolism were slower in diabetic than in normal rats. The results suggest that modulation of a carbohydrate-mediated glycoprotein recognition system located on hepatic endothelial cells can be induced by glucose and glucose-conjugated proteins and by fasting and diabetes mellitus. The findings in this study suggest a mechanism for abnormal glycoprotein metabolism in diabetes mellitus.

INTRODUCTION

The survival of glycoproteins in the circulation is critically influenced by the terminal nonreducing sugar residue of their oligosaccharide moiety (1). Mannose- and *N*-acetylglucosamine-terminated glycoproteins are cleared by the liver (2–7) and their removal is mediated by cells that line the hepatic sinusoids (8–14). The site of the mannose/*N*-acetylglucosamine glycoprotein recognition system has been alternately ascribed to either Kupffer (8, 10, 14) or endothelial cells (11). In this paper we provide evidence from studies of isolated rat hepatic cells that the mannose/*N*-acetylglucosamine receptor is present on hepatic endothelial cells. We also show that the ability of this receptor on endothelial cells to mediate the uptake of a specific ligand is potently inhibited in vitro by glucose and a glucose-albumin conjugate.

METHODS

Materials. Waymouth's medium dry powder, Hanks' balanced salt solution (HBSS)[1] complete and without Ca^{++}

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[1] *Abbreviations used in this paper*: AGOR, agalactoorosomucoid; ASOR, asialoorosomucoid; HBSS, Hanks' balanced salt solution.

and Mg^{++}, and horse serum were purchased from Gibco Laboratories, Grand Island Biological Co. (Grand Island, NY). HBSS without glucose was prepared as follows: $CaCl_2$ 0.14 g/liter, KCl 0.4 g/liter, KH_2PO_4 0.06 g/liter, $MgCl_2 \cdot 6H_2O$ 0.1 g/liter, $MgSO_4 \cdot 7H_2O$ 0.1 g/liter, NaCl 8.0 g/liter, $NaHCO_3$ 0.35 g/liter, $Na_2HPO_4 \cdot 7H_2O$ 0.09 g/liter, phenol red 0.01 g/liter. Collagenase type II (EC 3.4.24.3) was obtained from Worthington Biochemicals (Freehold, NJ). Deoxyribonuclease 1, type I (EC 3.1.4.5), soybean trypsin inhibitor type IIS, streptozotocin, glutaraldehyde, cacodylic acid, acid phosphatase staining kit, diaminobenzidine, bovine serum albumin, *p*-nitrophenyl-α-D-mannopyranoside, α-*N*-benzyloxycarbonyl-L-lysine P nitrophenyl ester-HCl, 2,6-dichlorophenol indophenol, β-nicotinamide adenine dinucleotide (reduced form; βNADH), mannan, D(+)mannose, *N*-acetyl-D-glucosamine, ovalbumin, β-D(−)fructose and D-glucose were obtained from Sigma Chemical Co. (St. Louis, MO). Percoll and density marker beads were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Silicone oils, dimethylpolysiloxanes 550 and 200/2 cs were obtained from Dow-Corning Co. (Corning, NY). Polystyrene microspheres for standardizing the apparatus for assessing cell size were from Coulter Electronics (Hialeah, FL). Carrier-free Na ^{125}I, $^{3}H_2O$, and [^{14}C]dextran were obtained from New England Nuclear (Boston, MA) and $Na^{36}Cl$ from Amersham-Searle (Arlington Heights, IL). Ultrafluor scintillation fluid was obtained from National Diagnostics (Somerville, NJ). Rabbit anti-whole sheep erythrocyte antibodies (7 and 19S) were obtained from Cordis Laboratories (Miami, FL). Sheep erythrocytes (E) were provided by the National Institutes of Health Animal Production Section. Pure undenatured human asialoorosomucoid (ASOR) and agalactoorosomucoid (AGOR) were generous gifts from Dr. Gilbert Ashwell. Orosomucoid had been isolated from normal human plasma and its terminal nonreducing carbohydrate residues had been modified by incubation with purified neuraminidase (to yield ASOR) or neuraminidase followed by purified β-D-galactosidase (to yield AGOR) (15). A neoglycoprotein, glucose-albumin conjugate (bovine serum albumin) was a gift from Dr. Y. C. Lee. It contained ~35 mol of glucose/mol albumin (16).

Iodination of glycoproteins. The procedure used to radioiodinate glycoproteins was the Bio-Rad Laboratories (Richmond, CA) solid-phase Enzymobead system, which uses immobilized lactoperoxidase and glucose oxidase. Rehydrated Enzymobead reagent, 25 μl, was incubated at room temperature for 25 min with 20 μl of protein (200 μg) in 0.1 M pH 6.8 phosphate buffer, 25 μl of 1% β-D-glucose, 50 μl of 1.0 M pH 6.8 phosphate buffer, and 10 μl of aqueous Na^{125}I containing a total of 1 mCi. The reaction was stopped by passing the mixture through G-25 Sephadex column (1.5 × 5 cm). The labeled protein was collected free of nonbound ^{125}I.

Animals. Male Sprague-Dawley rats (250–350 g) were purchased from Taconic Farms (Germantown, NY). Fed rats were allowed Purina rat chow (Ralston-Purina Co., St. Louis, MO) and water ad lib. Fasted rats were deprived of food for 24 h before liver cell isolation but were permitted unrestricted access to water. Diabetes mellitus was induced by the intravenous or intraperitoneal injection of streptozotocin (80 mg/kg in 0.09 M NaCl, 0.01 M citrate buffer, pH 3.5). Liver cell isolation was performed 4 d after streptozotocin administration. Blood glucose estimations were performed on blood collected into fluoride tubes by Litton Bionetics (Kensington, MD).

Liver perfusion. Animals were anesthetized with ether. Heparin was not used. Livers were perfused *in situ* using standard techniques (17, 18). Two rat livers were perfused simultaneously to increase the yield of endothelial and Kupffer cells. All perfusion fluids were gassed with 95% O_2:5% CO_2 and maintained at 37°C. Initially, HBSS without Ca^{++} and Mg^{++} was perfused (~700 ml) and the effluent discarded. The perfusate was then changed to 150 ml complete HBSS, containing collagenase (0.05% wt/vol) and soybean trypsin inhibitor (0.005% wt/vol). The trypsin inhibitor was added to reduce cell surface damage by proteases in the crude collagenase (19). The collagenase solution was recirculated for 30 min, and maintained at pH 7.4 by adding 1 M NaOH as required using phenol red as indicator. The resulting whole liver suspensions were dispersed in a further 50 ml of the collagenase solution to which deoxyribonuclease (0.0005% wt/vol) had been added and agitated in a water bath at 37°C for 10 min. Deoxyribonuclease (0.0005% wt/vol), which inhibits aggregation of cells in cell suspensions (20), was added to the buffer used in all subsequent steps of the isolation procedure. The cell suspension was filtered through 0.5-mm and 100-μm nylon sieves and the debris washed. The suspension was then made up to 100 ml before centrifugation at 20 *g* for 2 min at 21°C. The supernatant was decanted and the pellet resuspended in HBSS. This sequence of procedures was repeated twice. The final pellet consisting of parenchymal cells was resuspended in HBSS. The supernatants were also centrifuged twice more at 20 *g* for 2 min to remove contaminating parenchymal cells and were then centrifuged at 600 *g* for 7 min to yield a pellet containing nonparenchymal cells and some erythrocytes.

Viability of cells was assessed by two independent methods. Trypan blue exclusion was ≥80% for parenchymal cell enriched fractions and ≥90% for nonparenchymal cell enriched fractions. The membrane potential of isolated cells measured by the method described by Baur et al. (21), was −33±1.2 mV (mean±SEM, n = 12).

Cell purification on Percoll gradients. Density gradients of Percoll were selected for purification of the cell fractions because of the low toxicity of this medium (22, 23). The method adopted was as follows: Waymouth's medium dry powder (amount for 1 liter) was dissolved in 333 ml water and filtered through a 0.45-μm filter (Nalgene, Industrial Div. Nalge Co., Div. Sybron Corp., Rochester, NY). This solution was mixed with 667 ml Percoll and stored at 4°C. Before use deoxyribonuclease (0.0005% wt/vol) was added and the Percoll mixture gassed with 90% O_2:5% CO_2. The density gradients were generated by centrifuging 30-ml aliquots of the mixture in an SW 27 rotor in an L5-50 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 60,000 *g* for 1 h at 15°C. Suspensions of cells were layered on top of the gradients and centrifuged at 800 *g* for 15 min at 15°C. Using density marker beads, both endothelial and Kupffer cells were found to form a band at a density of 1.062 and parenchymal cells at a density of 1.097. As the two bands were located ~4 cm apart in the gradients, a clear separation of parenchymal and nonparenchymal cells was achieved. Cell debris and damaged cells settled at the top of the gradient (*d*, 1.033–1.048). The cells were recovered by aspirating separately each of the layers containing cells, diluting them 1:4 with HBSS and centrifuging the parenchymal cell fraction at 50 *g* for 2 min and the nonparenchymal cell fraction at 600 *g* for 7 min. Trypan blue exclusion was ≥95% for the parenchymal and nonparenchymal cells recovered from Percoll gradients.

Centrifugal elutriation. Centrifugal elutriation was used to separate endothelial from Kupffer cells (24, 25). The nonparenchymal cells, suspended in HBSS (8 ml), were loaded into a Beckman JE6-B elutriator rotor in a J2-21 centrifuge at 15°C. Elutriation was carried out at a rotor speed of 2,555 rpm. The rotor was initially eluted with HBSS at a flow rate

of 11 ml/min. At this flow rate 150 ml buffer were collected and discarded to remove debris and lymphocytes. The flow rate was increased to 21 ml/min and 100 ml buffer collected. This fraction contained predominantly endothelial cells. The flow rate was increased to 42 ml/min and a further 100-ml buffer was collected. This fraction contained predominantly Kupffer cells. The cells were recovered by centrifuging the eluates at 600 *g* for 7 min. Cells were counted in a Coulter counter model ZH and their size estimated using a Coulter channelyser C1000 with a log range expander. The channelyser was calibrated with polystyrene microspheres of Diam 9.86 and 19.54 μm.

Histochemical stains. Suspensions of cells from the endothelial and Kupffer cell fractions were stained for endogenous peroxidase with 1% (wt/vol) diaminobenzidine, 0.02% (vol/vol) H_2O_2 in 0.1 M Tris–HCl (pH 7.6) at 21°C for 30 min (26). Smears of the stained cells were air dried and were then fixed in a buffer containing 1.5% (vol/vol) glutaraldehyde, 1% (wt/vol) sucrose and 0.067 M cacodylic acid (pH 7.4). Tartrate-resistant acid phosphatase was detected in air-dried smears of cells that had been fixed with a sodium citrate/ acetone solution (pH 5.4) using napthol AS-B1 phosphate and fast garnet GBC (Sigma kit). All the slides were counterstained with hematoxylin and at least 200 cells were counted on each slide.

Enzyme estimations. Measurements of the activities of cell enzymes were made on samples of cells from endothelial and Kupffer cell fractions that had been frozen at −20°C and thawed. α-D-mannosidase was estimated using *p*-nitrophenyl-α-D-mannopyranoside as a substrate (27). Cathepsin B_1 (EC 3.4.4.-) was estimated using α-*N*-benzyloxycarbonyl-L-lysine-*p*-nitrophenyl ester-HCl (28). Lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) (29) and succinate dehydrogenase (30) were also measured. Reaction rates were determined by measurements of optical density using a Beckman Acta III spectrophotometer. Total protein was estimated by a modified Lowry technique (31).

Fc and C3b receptors. Fc and C3b receptors on cells from the endothelial and Kupffer cell fractions were detected by determining their ability to form rosettes when incubated with IgG (7S) (EA), and IgM (19S) and complement-sensitized (EAC) sheep erythrocytes, respectively (32). Fresh serum from C57B mice was used as a source of complement. As controls cells were incubated in parallel with E alone or E and IgM (19S) without complement. Rosettes were defined as three or more erythrocytes adhering to an hepatic cell.

Flow cytometry. Flow cytometry was performed in a Coulter TPS1 cell sorter (33).

Ligand-cell binding assay. Suspensions of cells containing labeled ligand and/or inhibitors (2 ml) were incubated at 37°C with agitation in glucose free HBSS that had been gassed with 95% O_2:5% CO_2. The concentration of endothelial and Kupffer cells in the suspensions was $\sim 5 \times 10^6$ cells/ml and the concentration of parenchymal cells $\sim 5 \times 10^5$ cells/ml. Following addition of a labeled ligand (sp act 3–4 μCi/μg) to a cell suspension, samples (250 μl) were removed and placed in 400-μl microfuge tubes that already contained 100 μl silicone oil (34) (*d*, 1.03; prepared by mixing 85.6 g 550 oil with 17.4 g 200/2 cs oil) on top of a denser layer of 50 μl 3 M KOH. The microfuge tubes were centrifuged in a Beckman microfuge B for 30 s. The cell pellet in the KOH layer was removed by slicing through the silicone oil layer and radioactivity in the pellet measured using a Nuclear Chicago gamma counter (Nuclear Chicago Corp., Des Plaines, IL). Acid precipitable radioactivity in the microfuge tube supernatant was also determined by adding to 200 μl of the supernate horse serum (800 μl) and 25% (wt/vol) trichloroacetic acid (1 ml), centrifuging at 3,500 *g* for 7 min, and measuring the radioactivity in the supernate and pellet. Specific uptake of ^{125}I-AGOR by cells was estimated by subtracting its uptake in the presence of the inhibitor mannan (100 μg/ml), a polysaccharide of mannose, from that in the absence of inhibitor. In all experiments nonspecific uptake accounted for <20% of maximal uptake.

In vivo studies. A tracer dose of ^{125}I-AGOR (~ 2 μg; 5×10^6 cpm) was injected under ether anesthesia into a femoral vein. Blood samples (0.5 ml) were obtained by repeated cardiac puncture during the subsequent 0.5–7 min. The liver, spleen, and left kidney were then rapidly removed and their radioactivity content estimated. The blood disappearance curve of ^{125}I-AGOR was defined by determining the best fit of experimental measurements of blood acid precipitable radioactivity to the sum of exponential functions using a DEC 10 computer. From the parameters of the fitted curve the percentages of the initial radioactivity remaining in the blood at seven successive 1-min intervals after the injection of ^{125}I-AGOR were calculated. The mean of values for protein-bound radioactivity (±SEM) at each of these selected times was calculated for each different experimental group (see below). To determine the fractional disappearance rate of ^{125}I-AGOR from blood the fitted blood radioactivity curves were normalized so that the sum of the coefficients of the exponentials equalled unity. The fractional disappearance rate was derived by determining the reciprocal of the area under the normalized curve, which is equivalent to $1 \Big/ \sum_{j=1}^{n} \frac{A_j}{g_j}$ where A_1, $A_2 \ldots A_n$ are the coefficients and g_1, $g_2 \ldots g_n$ the rate constants of the exponentials that define the curve (35) The apparent initial volume of distribution of ^{125}I-AGOR was estimated by the principle of isotope dilution, using the extrapolated value for blood radioactivity at zero time generated from the computer fit of the blood radioactivity data.

RESULTS

Characterization of isolated cell populations

On average, nonparenchymal cells accounted for 42% of the total cells recovered from whole liver cell suspensions; endothelial cells accounted for 75% and Kupffer cells 25% of recovered nonparenchymal cells. Properties of cells in isolated endothelial and Kupffer cell fractions are shown in Table I. Histochemical stains for endogenous peroxidase and tartrate-resistant acid phosphatase, enzymes that are considered to be restricted to Kupffer cells (36, 37), indicated that ~9% of cells in the endothelial cell fraction were Kupffer cells and that ~87% of cells in the Kupffer cell fraction were Kupffer cells. Flow cytometry indicated that the fraction of Kupffer cells in the growth phase was about three times greater than that for endothelial cells indicating that Kupffer cells are a rapidly proliferating cell population.

Effect of fasting on isolated cell populations

A 24-h fast had profound effects on both the recovery and properties of isolated hepatic cells. The yield of endothelial cells from fasted rats ($81 \pm 5 \times 10^6$ cells)

TABLE I
Properties of Cells in Endothelial and Kupffer Cell Fractions

	n*	Endothelial‡	Kupffer‡
Morphology			
Cell recovery, $\times 10^6$ *per 2 rats*	31	198.0±14.0	62.0±4.0
Modal cell volume, μm^3	33	130.0±5.0	248.0±6.0
Protein content, $\mu g/10^6$ *cells*	8	65.0±4.0	149.0±15.0
Histochemistry			
Endogenous peroxidase (percent cells positive)	4	8.0±2.0	88.0±2.0
Acid phosphatase (percent cells positive)	9	9.0±1.0	86.0±2.0
Immunology	6		
Fc receptors (percent cells forming EA rosettes)		8.0±1.0	53.0±6.0
C3b receptors (percent cells forming EAC rosettes)		2.0±2.0	20.0±1.0
Flow cytometry	6		
G1 (resting phase), %		97.0±0.4	91.0±0.8
S + G2M (growth phase), %		2.6±0.4	8.9±0.8

* *n*, number of cell isolations.
‡ Mean±SEM.

was ~60% lower than that from fed rats (198±14 × 10^6 cells). A smaller reduction (32%) in the yield of Kupffer cells also occurred on fasting (fed, 62±4 × 10^6 cells; fasted, 42±3 × 10^6 cells). However, the yield of cells in the parenchymal fraction was 100 × 10^6 cells (20%) greater in fasted than in fed animals. Consequently, the total cell yield in fasted rats (595±32 × 10^6 cells) was not significantly different from that in fed rats (631±31 × 10^6 cells).

Fasting was associated with a decrease in body weight of 3±1.5% (n = 4) but a decrease in liver weight of 23±3%. The magnitude of this decrease in liver weight corresponded to the decrease (27%) in the modal volume of isolated parenchymal cells (fed, 3,218±108 μm^3, n = 33; fasted, 2,334±60 μm^3, n = 30). In contrast the modal cell volume of isolated Kupffer cells from fasted rats (525±17 μm^3, n = 30) was much greater than that from fed rats (248±6 μm^3, n = 30). These volumes indicate that the diameter of Kupffer cells was 10.1±0.11 μM in fasted rats and 7.8±0.06 μM in fed rats. Distribution plots of cell size indicated the presence of two subpopulations of Kupffer cells. Yields of Kupffer cells of both the large and small subpopulations were appreciable from fed animals but cells predominantly of the larger subpopulation were isolated from fasted animals. The modal cell volume of isolated endothelial cells was not affected by fasting.

Data on the protein content of cells, the proportion of cells stained for tartrate-resistant acid phosphatase and cell cytometry (data not shown) were similar for endothelial cells isolated from both fed and fasted animals and for Kupffer cells isolated from both fed and fasted animals. Furthermore, fasting did not influence the activity of the lysosomal enzymes α-D-mannosidase and cathepsin B_1 or the cytosolic enzyme lactate dehydrogenase. However, fasting was associated with a significant reduction (~33%) in the activity of the mitochondrial enzyme, succinate dehydrogenase.

Effect of streptozotocin-induced diabetes mellitus on isolated cell populations

Diabetes mellitus was associated with a reduction in the mean yield of endothelial cells (100±9 × 10^6 cells), the yield of these cells being similar to that obtained from fasted normal rats. The recoveries of Kupffer cells (52±3 × 10^6 cells) and parenchymal cells (337±32 × 10^6 cells) from diabetic rats were similar to corresponding recoveries from fed normal rats. Consequently, the total yield of hepatic cells from diabetic animals (490±36 × 10^6 cells) was ~100 × 10^6 cells less than that from fed normal rats. This difference was accounted for by the lower recovery of endothelial cells (~98 × 10^6 fewer) from diabetic rats. In diabetic animals a 24-h fast was not associated with any appreciable further changes in the recoveries of different cells.

Uptake of glycoproteins by hepatic cells

Uptake of ^{125}I-AGOR by hepatic cells from fed and fasted rats. The uptake of ^{125}I-AGOR (*N*-acetylglucosamine-terminated) by cells in preparations of endothelial cells and Kupffer cells from fed rats was time-dependent at 37°C (Fig. 1). Uptake of ^{125}I-AGOR by

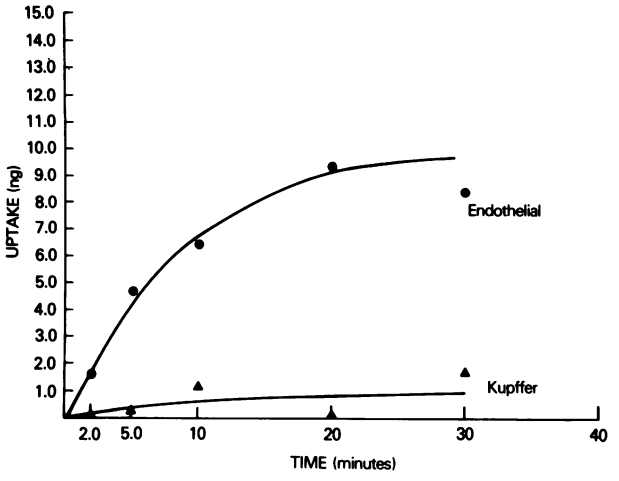

FIGURE 1 The time dependence of the specific uptake of ^{125}I-AGOR (2,000 ng/ml) at 37°C by endothelial and Kupffer cells (5×10^6/ml) isolated from fed rats. Specific uptake was calculated by subtracting from total uptake the uptake of ligand in the presence of the inhibitor mannan (100 μg/ml). Uptake of ^{125}I-AGOR by endothelial cells was appreciable. Uptake of the labeled ligand by cells in preparations of Kupffer cells was much less than that by endothelial cells. It varied with different preparations and could have been due, at least in part, to variable contamination of the Kupffer cell fractions with endothelial cells. Appreciable catabolism of ^{125}I-AGOR (as measured by the accumulation of acid soluble radioactivity) was not detected after 20 min incubation and was only up to 5% of the added radioactivity after 30 min incubation. The results of this experiment are representative of three other similar experiments.

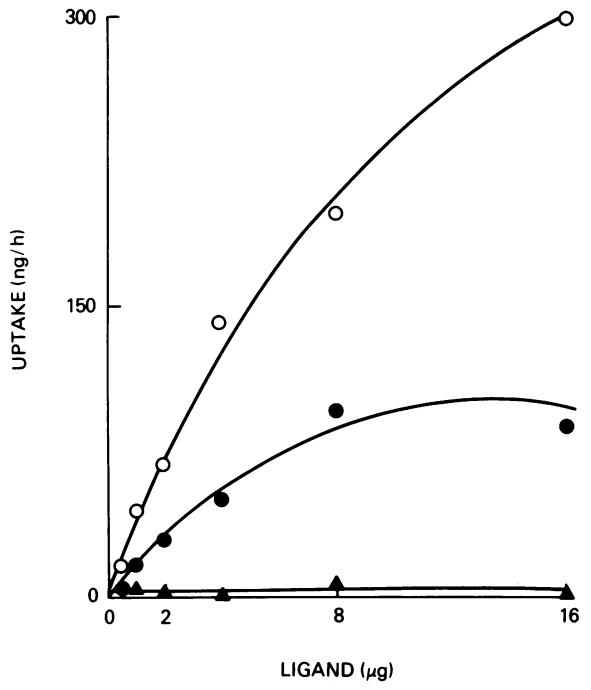

FIGURE 2 The concentration dependence of the specific uptake of ^{125}I-AGOR by endothelial cells. The data shown were obtained using endothelial cells isolated from fasted (○) and fed rats (●), and Kupffer cells (fasted or fed) (▲). Uptake velocity (nanograms per hour) was calculated from a least-squares fit of the uptake data at 2, 5, 10, and 20 min after addition of the labeled ligand. The data points represent the mean values from 6 (○), 5 (●), and 3 (▲) experiments. The uptake of labeled ligand by endothelial cells was appreciable. It approached saturation at high substrate concentrations and was enhanced by a 24-h fast before cell isolation. In contrast, Kupffer cells took up little ^{125}I-AGOR.

endothelial cells was appreciable. In contrast, uptake of ^{125}I-AGOR by cells in preparations of Kupffer cells was consistently much lower than that by endothelial cells; it varied for different preparations and could have been due, at least in part, to variable contamination of the Kupffer cell fraction with endothelial cells (see above). The uptake of ^{125}I-AGOR by endothelial cells and by cells in preparations of Kupffer cells was directly proportional to the number of cells present; that is, it was protein dependent. Uptake of ^{125}I-AGOR by parenchymal cells was negligible. Furthermore, in contrast to parenchymal cells, the in vitro uptake of ^{125}I-ASOR (galactose-terminated) by endothelial and Kupffer cells was negligible (data not shown). When ^{125}I-AGOR was incubated with nonparenchymal cells acid soluble radioactivity in the medium did not increase during the first 20 min but began to accumulate thereafter and was up to 5% of the added radioactivity at 30 min, indicating some cell-mediated catabolism of this labeled ligand.

The concentration dependence of the in vitro uptake of ^{125}I-AGOR by endothelial and Kupffer cells at 37°C is shown in Fig. 2. The data demonstrate that uptake of this labeled ligand by endothelial cells is appreciable and is much greater by endothelial cells isolated from fasted rats than by those isolated from fed rats. The uptake of ^{125}I-AGOR by endothelial cells from fed animals approached saturation at high substrate concentrations. In contrast, the uptake of ^{125}I-AGOR by cells in Kupffer cell fractions was minimal. Double reciprocal plots of the initial uptake velocity and the ligand concentration showed that the uptake of ^{125}I-AGOR by endothelial cells from both fasted and fed animals could be described by Michaelis-Menten kinetics (Fig. 3). The effect of fasting was to increase the apparent maximum velocity about threefold (fed, 4.8 pmol/h per 5×10^6 cells; fasted, 14.3 pmol/h per 5×10^6 cells) while the apparent K_m remained constant (0.29 μM).

Inhibition of the uptake of ^{125}I-AGOR by endothelial cells. In preliminary experiments, glucose was found to inhibit the in vitro uptake of ^{125}I-AGOR by endothelial cells. Consequently, all incubations, unless

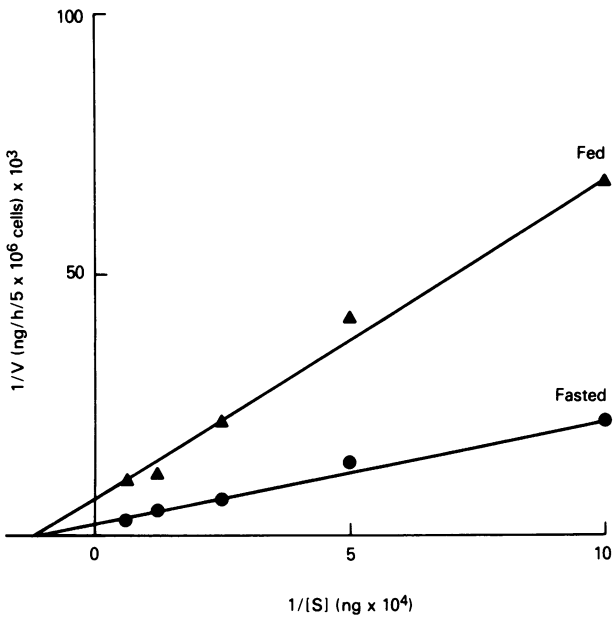

FIGURE 3 The effect of fasting on the specific uptake of ^{125}I-AGOR by endothelial cells. Double reciprocal plots of mean values for uptake by endothelial cells from fed (▲) and fasted (●) rats are shown (data in Fig. 2 replotted). Fasting did not alter the apparent K_m (0.29 μM) but the apparent maximum velocity increased approximately threefold (fed, 4.8 pmol/h per 5×10^6 cells; fasted, 14.3 pmol/h per 5×10^6 cells). These findings indicate that fasting does not alter the affinity but it increases the density of mannose/*N*-acetylglucosamine receptors on endothelial cells. S, ligand concentration; V, velocity.

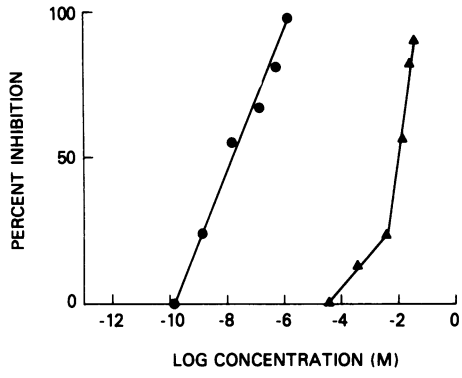

FIGURE 4 Glucose-mediated inhibition of the uptake of ^{125}I-AGOR by endothelial cells. Data on the uptake of ^{125}I-AGOR (14.3 nM, 35,000 daltons) by endothelial cells from fasted rats in the presence of different concentrations of a glucose-albumin conjugate (●), or different concentrations of glucose (▲) are shown. The glucose-albumin conjugate (67,000 daltons) contained 35 mol of glucose per mole of albumin. The calculation of the uptake velocity of ^{125}I-AGOR is described in the legend to Fig. 2 and inhibition of uptake was determined as described in the footnote to Table II. Uptake of ^{125}I-AGOR was progressively inhibited by increasing glucose concentrations in the range 0.56 to 56 mM (10–1,000 mg/dl). The glucose-albumin conjugate was six orders of magnitude more potent an inhibitor (on a molar basis) than glucose alone. One experiment was performed with glucose-albumin conjugate. Each data point for glucose inhibition represents the mean of two experiments.

otherwise stated, were performed in glucose-free HBSS. Furthermore, to examine factors that inhibit the uptake of ^{125}I-AGOR by endothelial cells under optimal conditions, unless otherwise stated, endothelial cells isolated from fasted rats were used. Data showing that glucose inhibits the uptake of ^{125}I-AGOR by endothelial cells are shown in Fig. 4. The data reveal that inhibition of uptake occurs in the presence of glucose concentrations in the physiological range and is virtually 100% in the presence of glucose concentrations that may be encountered in diabetes mellitus. At a glucose concentration of 100 mg/dl (5.6 mM) the uptake of ^{125}I-AGOR was reduced ~23% and at 1,000 mg/dl (56 mM) it was reduced ~90%. Double reciprocal plots of data on the uptake of ^{125}I-AGOR by endothelial cells in the presence and absence of glucose are shown in Fig. 5. The data indicate that glucose competitively inhibits the uptake of ^{125}I-AGOR by endothelial cells. A glucose concentration of 500 mg/dl (28 mM) did not affect the apparent maximum velocity (14.3 pmol/h per 5×10^6 cells) but it increased the apparent K_m (1.1 μM) nearly fourfold.

Fig. 4 also shows that the uptake of ^{125}I-AGOR by endothelial cells is inhibited by the neoglycoprotein, glucose-albumin conjugate. The glucose-albumin conjugate was six orders of magnitude more potent an

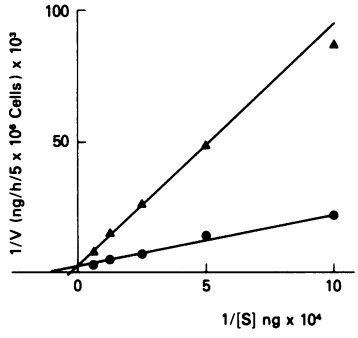

FIGURE 5 The effect of glucose on the uptake of ^{125}I-AGOR by endothelial cells isolated after fasting. Double reciprocal plots of the mean uptake of ^{125}I-AGOR by endothelial cells isolated from fasted rats in the presence of glucose (▲) (28 mM; 500 mg/dl) and in the absence of glucose (●) are shown. The data points for uptake in the presence of glucose represent the mean of eight experiments. Corresponding data for uptake in the absence of glucose are from Fig. 3. The data indicate that glucose competitively inhibits ^{125}I-AGOR uptake by endothelial cells. Glucose did not influence the apparent maximum velocity (14.3 pmol/h per 5×10^6 cells) but increased the apparent K_m (1.1 μM) nearly fourfold. S, ligand concentration; V, velocity.

inhibitor of this uptake process than glucose alone. Furthermore, 50% inhibition of the uptake of ^{125}I-AGOR (14.3 nM) occurred in the presence of 15 nM glucose-albumin conjugate suggesting that these two glycoproteins had identical binding constants. Table II summarizes data on the inhibition of endothelial cell uptake of ^{125}I-AGOR by a variety of compounds. Uptake was inhibited by the monosaccharides, *N*-acetylglucosamine, glucose, mannose, and fructose. Uptake was also inhibited by the glycoproteins agalactoorosomucoid (*N*-acetylglucosamine-terminated) and the glucose-albumin conjugate and by the polysaccharide of mannose, mannan, but it was not inhibited by the glycoprotein asialoorosomucoid (galactose-terminated). Uptake was partially inhibited by ovalbumin, a mannose-terminated glycoprotein that exhibits microheterogeneity (38). The uptake of ^{125}I-AGOR by endothelial cells was abolished in the presence of EDTA indicating that this uptake process has an absolute requirement for calcium ions. Furthermore, the uptake of ^{125}I-AGOR by endothelial cells at 0°C (assumed to be due to binding only) was less than that at 37°C (Table II) and no catabolism of ^{125}I-AGOR (accumulation of acid-precipitable radioactivity in the incubation medium) was observed at 0°C. These observations imply that at 37°C both binding and internalization of ^{125}I-AGOR by endothelial cells occur.

Effect of streptozotocin-induced diabetes mellitus on the uptake of ^{125}I-AGOR by endothelial cells. In view of the marked inhibition of the endothelial cell uptake of ^{125}I-AGOR by glucose and the observation that fasted animals not only had a lower blood glucose concentration (120±3 mg/dl; n = 11) than fed animals (138±5 mg/dl; n = 6) but also exhibited augmented uptake of ^{125}I-AGOR by isolated endothelial cells, experiments were designed to determine whether hyperglycemia influenced the enhancement of ^{125}I-AGOR uptake induced by fasting. Endothelial cells were isolated from rats with streptozotocin-induced diabetes mellitus. Some diabetic rats were fasted for 24 h before isolation of the cells. The blood glucose concentration in fed diabetic rats was 517±44 mg/dl (n = 8) and in fasted diabetic rats was 323±29 mg/dl (n = 5). The uptake of ^{125}I-AGOR by endothelial cells, isolated from fed diabetic rats (in glucose-free HBSS) was similar to that by cells isolated from fed normal rats. Furthermore, in diabetic rats fasting did not result in enhancement of endothelial cell uptake of ^{125}I-AGOR. Thus, the apparent maximum velocity (4.8 pmol/h per 5×10^6 cells) and the apparent K_m (0.29 μM) for ^{125}I-AGOR uptake by endothelial cells isolated from both fed and fasted diabetic animals were similar to the corresponding values obtained for normal fed rats.

TABLE II
Inhibition of ^{125}I-AGOR Uptake by Endothelial Cells In Vitro

Inhibitor	Concentration	Inhibition*
		%
Monosaccharides		
N-acetylglucosamine	50 mM	70
Fructose	50 mM	100
Mannose	50 mM	89
Glucose	50 mM	96
Glycoproteins		
Asialoorosomucoid	100 μg/ml	0
Ovalbumin	100 μg/ml	54
Agalactoorosomucoid	100 μg/ml	94
Glucose-albumin conjugate	100 μg/ml	98
Miscellaneous		
Mannan	100 μg/ml	100
EDTA	7.5 mM	99
O°C		71

* Inhibition was expressed as the percentage reduction of the initial uptake velocity of ^{125}I-AGOR (500 ng/ml) by an inhibitor. ^{125}I-AGOR was incubated with endothelial cells in the presence and absence of inhibitors at 37°C. These results are representative of two other similar experiments. The initial uptake velocity was calculated from a least-squares fit of the uptake data at 2, 5, 10, and 20 min after addition of the labeled ligand.

In vivo metabolism of ^{125}I-AGOR. The in vivo metabolism of ^{125}I-AGOR was studied in groups of fed, fasted, and diabetic rats. The sum of two exponential functions was found to be both necessary and sufficient to define the plasma disappearance curves of intravenously administered ^{125}I-AGOR. Apparent initial volume of distributions of ^{125}I-AGOR (~25 ml) approximated to the blood volume (39) and were similar in fed, fasted and diabetic rats (Table III). The plasma disappearance of ^{125}I-AGOR (Fig. 6) in fed rats was rapid. In fasted rats, the mean plasma disappearance was more rapid. However, the mean fractional disappearance rates for fed and fasted animals were not significantly different (Table III). In both fed and fasted animals, 7 min after injection of ^{125}I-AGOR ~66% of the administered radioactivity had accumulated in the liver and <2% was found in the spleen or kidney. By 7 min ~12% of the administered ^{125}I-AGOR had been catabolized, as determined by accumulation of acid soluble radioactivity in blood. In contrast, in diabetic animals (blood glucose concentration 438±12 mg/dl) the fractional plasma disappearance rate, hepatic accumulation (51%), and catabolism (4.3%) of ^{125}I-AGOR were all less than in either fed or fasted normal rats (Table III).

DISCUSSION

Isolation of hepatic cells. The objectives of the cell isolation procedures used in this study were to prepare

TABLE III
In Vivo Metabolism of ^{125}I-AGOR

	n	Apparent initial volume of distribution	Fractional disappearance rate	Distribution*			Catabolism‡
				Liver	Spleen	Kidney	
		ml	*per min*				%
Fed	5	25.4±0.8	0.36±0.06	65±2	1.1±0.1	1.6±0.3	12.0±1.0
Fasted	7	24.5±1.0	0.42±0.07	67±1	0.8±0.1	1.2±0.1	11.0±2.0
Diabetic	3	25.4±0.9	0.20±0.02	51±2	0.4±0.1	2.5±0.2	4.3±0.3

* Distribution is expressed as percent injected radioactivity accumulating in an organ by 7 min.
‡ Catabolism is expressed as the percentage of the injected dose that appeared as acid-soluble radioactivity in blood by 7 min.

purified preparations of functionally competent endothelial, Kupffer and parenchymal cells from rat liver. The refinements incorporated into the isolation methodology (19, 20, 22, 23) resulted in the generation of populations of cells that contained a high proportion of cells that excluded trypan blue and had an adequate mean membrane potential (21). Although some cell surface damage may have occurred during the isolation of cells (40), it would be most unlikely that the isolation procedures would have selectively altered glycoprotein receptors on one cell type and not on another. It is noteworthy that the large Kupffer cell growth fraction provides strong support for the view that in normal rats Kupffer cells are a locally self-replicating population and are not replenished from outside the liver (for review, see 41).

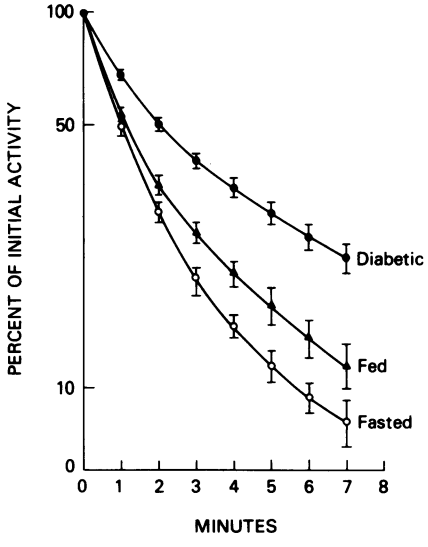

FIGURE 6 The metabolism of ^{125}I-AGOR in vivo. Mean plasma disappearance curves of an intravenously administered tracer dose (~2 μg) of ^{125}I-AGOR in fed normal (▲: n = 5), fasted normal (○: n = 7) and diabetic rats (●: n = 3) are shown. The plasma disappearance rate of ^{125}I-AGOR tended to be higher in fasted normal rats and was significantly slower in diabetic rats than in fed normal rats.

Fasting appeared to prevent the isolation of a proportion of sinusoidal cells from whole liver cell suspensions. It also may have induced an increase in the size of Kupffer cells or alternatively, since subpopulations of Kupffer cells of different sizes were isolated from fed animals, it is possible that fasting resulted in the selective isolation of a subpopulation of large Kupffer cells. Heterogeneity of the size of Kupffer cells isolated from fed rats has been reported previously (42). The causes of the reduced sinusoidal cell recoveries from the livers of fasted rats are obscure. Endothelial and Kupffer cells are not attached to parenchymal cells by formal structures such as desmosomes (43–45) so that is is possible that the reduced recovery of sinusoidal cells is attributable to stronger cell-cell interactions between sinusoidal and parenchymal cells after fasting.

Streptozotocin (2-deoxy-2-[3-methyl-3-nitrosourea]-D-glucopyranose) is believed to be a relatively specific toxin for pancreatic beta cells (for reviews see 46, 47). It has been suggested that the glucose moiety of the streptozotocin molecule mediates its specific recognition and uptake by pancreatic beta cells. In support of this hypothesis are the observations that the diabetogenic effectiveness of streptozotocin is reduced by removal of the glucose moiety or its substitution by galactose or by the administration of certain monosaccharides such as glucose, 3-*O*-methyl glucose and 2-deoxyglucose (47). In view of the findings in this study that show that the mannose/*N*-acetylglucosamine receptors on endothelial cells also possess specificities for glucose and glucose-terminated glycoproteins (discussed below), it is possible that the reduced recovery of endothelial cells from streptozotocin-treated rats is due to a direct toxic effect of streptozotocin on these cells. Such an effect could have important implications for investigations of lipid meta-

bolism and the vascular complications of diabetes mellitus using the streptozotocin animal model. An alternative explanation, that diabetes mellitus has a direct effect on endothelial cell recovery, cannot be discounted.

Uptake of glycoproteins by hepatic cells. This study has confirmed previous observations that ^{125}I-AGOR, an *N*-acetylglucosamine-terminated glycoprotein, is rapidly cleared from plasma by the liver (2–7, 9). It is possible that the receptor that mediates this clearance process and a mannan-binding protein, which has been isolated from whole liver (48), are identical. This study has also confirmed that, in contrast to galactose-terminated glycoproteins (12), the hepatic clearance of ^{125}I-AGOR is mediated by sinusoidal cells and not parenchymal cells (8, 10, 12, 13). On the basis of extrapolations from circumstantial evidence (14, 49) it has been presumed that the mannose/*N*-acetylglucosamine receptor is located on Kupffer cells. However, the only previous study to have directly addressed the question of the localization of the mannose/*N*-acetylglucosamine receptor indicated that it was present principally on hepatic endothelial cells (11). In that study, which used both autoradiography and electron microscopy, the label of intravenously injected ^{125}I-labeled mannose and *N*-acetylglucosamine-terminated glycoproteins was shown to accumulate selectively in hepatic sinusoidal cells and endothelial cells were found to internalize two to six times more radioactivity (on a cell volume basis) than Kupffer cells. Our in vitro data clearly indicate that the mannose/*N*-acetylglucosamine receptor is located on hepatic endothelial cells and that AGOR not only binds to this receptor but is also internalized by these cells.

This study has confirmed the previously reported dual specificity of the receptor for glycoproteins on hepatic sinusoidal cells (13). However, uptake of AGOR by endothelial cells was not only inhibited by *N*-acetylglucosamine and mannose, but also by glucose and its isomer fructose, indicating that the mannose/*N*-acetylglucosamine receptor possesses additional specificities. The failure of previous studies to detect inhibition of sinusoidal cell uptake of AGOR by glucose is probably attributable to either administering too little glucose during in vivo studies (8) or to performing in vitro experiments in a medium containing high concentrations (500 mg/dl) of glucose (13). The results of this study indicate that glucose competitively inhibits the uptake of AGOR by the mannose/*N*-acetylglucosamine receptor over a concentration range of two orders of magnitude that includes both physiological and pathological concentrations. In the presence of high glucose concentrations, similar to those that may occur in plasma in diabetes mellitus, almost complete inhibition of the uptake of AGOR by endothelial cells was observed. It is noteworthy that a glucose-terminated glycoprotein (glucose-albumin conjugate) was about six orders of magnitude more potent an inhibitor of the uptake of AGOR by endothelial cells than glucose alone. In other carbohydrate-mediated glycoprotein recognition systems similar differences in the potency of glycoproteins and relevant simple sugars alone in inhibiting the binding of a specific ligand are well recognized (50). Glucose has also been shown to inhibit the binding of an *N*-acetylglucosamine-bovine serum albumin conjugate to the mannose/*N*-acetylglucosamine receptor in vitro (51). The inhibition of the endothelial cell uptake of AGOR by fructose is of particular interest because a fructose residue is the terminal sugar of spontaneously glycosylated proteins such as hemoglobin A_{1C} and glycosylated albumin. The terminal fructose residue of such glycoproteins is generated by an Amadori rearrangement of the Schiff base formed between the free amino groups of the protein and glucose (52). However, the capacity of glycoproteins, such as the glucose-albumin conjugate, to inhibit the uptake of specific ligands by glycoprotein recognition systems depends largely on the degree of substitution of the protein with sugar residues (53). At present, there is no evidence that sufficient glycosylation of proteins occurs in diabetes mellitus to mediate uptake of glycosylated proteins by endothelial cells. Conversely, the accumulation of glycosylated proteins in plasma in diabetes mellitus (54) could be due, at least in part, to hyperglycemia inhibiting their uptake by hepatic endothelial cells.

The interpretation of the results of the in vitro experiments is complicated by the variations in endothelial cell yield in the different experimental groups. Although fasting was associated with a threefold increase in the uptake of ^{125}I-AGOR by endothelial cells without any change in the affinity of the receptor on these cells, the yield of endothelial cells from fasted animals was almost three times lower than that from fed rats. Two interpretations of these findings are possible. First, the observed augmented uptake of AGOR could be due to a fasting-induced up-regulation of mannose/*N*-acetylglucosamine receptors on endothelial cells. Second, a subpopulation of endothelial cells enriched with these receptors may have been selectively isolated from fasted animals. This second possibility tends to be favored by the observation that the plasma disappearance and hepatic accumulation of ^{125}I-AGOR in vivo were similar in fed and fasted animals.

The results of the in vitro experiments indicated that the induction of diabetes mellitus did not influence the uptake of ^{125}I-AGOR by isolated endothelial cells in a glucose-free medium and, in contrast to the data

obtained from normal animals, that prior fasting of diabetic animals did not augment the uptake of this ligand by isolated endothelial cells. Potential explanations of the latter finding include an abolition of fasting-induced up-regulation of the mannose/*N*-acetylglucosamine receptor due to hyperglycemia or some other effect of diabetes mellitus and a direct toxic effect of streptozotocin on a subpopulation of endothelial cells enriched with mannose/*N*-acetylglucosamine receptors (see above). As it is uncertain whether streptozotocin is toxic to hepatic endothelial cells caution is necessary in the interpretation of the decreased clearance from plasma, hepatic accumulation, and catabolism of ^{125}I-AGOR observed in diabetic animals. However, it has been shown that the clearance of an *N*-acetylglucosamine-bovine serum albumin conjugate from plasma in mice is diminished if diabetes mellitus is induced using alloxan, and that this phenomenon also occurs if hyperglycemia is induced in the absence of diabetes mellitus (51).

In conclusion, this study has demonstrated that a specific carbohydrate-mediated glycoprotein recognition system is present on hepatic endothelial cells. Thus, these cells appear to possess functional specialization in addition to their morphological specializations such as sieve plates (43). We have also shown that the receptor-mediated uptake by endothelial cells of a glycoprotein terminating in *N*-acetylglucosamine is markedly inhibited by glucose and by a glucose-albumin conjugate. Glucose and glycosylated protein-induced modulation of receptor-mediated pinocytosis of glycoproteins by vascular endothelial cells may contribute to abnormal metabolism of glycoproteins in diabetes mellitus.

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