Evidence for surface glycoprotein involvement in the intracellular bioactivity of insulin in rat adipocytes

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Lectins specific for D-mannose (concanavalin A), N-acetyl-D-glucosamine (wheat-germ agglutinin) or D-galactose (*Ricinus communis* agglutinin I) inhibited insulin binding and activated glucose transport in rat adipocytes [Cherqui, Caron, Capeau & Picard (1982) *Mol. Cell. Endocrinol.* **28**, 627–643]. In the present investigation, the intracellular activities of insulin and lectins on lipogenesis and protein synthesis were studied under conditions where neither agent had an effect on membrane transport processes. (1) When glucose transport was rate-limiting (0.5 mM-glucose), insulin (0.8 ng/ml) and lectins (20 µg/ml) increased lipogenesis by 2.4–3-fold. (2) When passive diffusion of glucose was amplified (10 mM-glucose), insulin (0.8 ng/ml) and lectins (20 µg/ml) increased lipogenesis by 1.6–1.8-fold even in the presence of 50 µM-cytochalasin B, which completely blocked glucose transport. (3) Insulin (6 ng/ml), concanavalin A and wheat-germ agglutinin (40 µg/ml) stimulated the incorporation of L-[U-¹⁴C]leucine into fat-cell protein 1.5-fold but did not modify α-aminoisobutyric acid uptake or ¹⁴C-labelled protein degradation. (4) Peanut and soya-bean agglutinins (specific for O-glycosidically-linked oligosaccharides), known not to alter insulin binding, were ineffective. (5) Lectin effects were dose-dependent and were markedly inhibited by specific monosaccharides (50 mM). (6) Insulin and lectin maximal effects were not additive and were completely abolished by neuraminidase treatment of fat-cells (0.05 unit/ml). These data indicate involvement of surface sialylated glycoproteins of the complex N-linked type in the insulin stimulation of glucose and amino acid intracellular metabolic processes. They suggest, together with our previous results, that the transmission of the insulin signal for both membrane and intracellular effects occurs via glycosylated effector entities of, or closely linked to, the insulin–receptor complex.

Much progress has been made recently in defining the structural and chemical features of the insulin receptor. The native receptor has been proposed to consist of two α-receptor subunits (M_r 135 000) and two β-receptor subunits (M_r 95 000); all are disulphide-linked (Massague *et al.*, 1980) and contain sialylated glycosidic moieties of the complex N-linked type (Hedo *et al.*, 1981). The involvement of such carbohydrate components in the first step of the action of insulin, i.e. insulin binding to the plasma-membrane receptor, has been reported in several studies (Cuatrecasas, 1971, 1973; Capeau *et al.*, 1978; Caron *et al.*, 1978; Sandra *et al.*, 1979; Capeau & Picard, 1980; Herzberg *et al.*, 1980;

Caron & Picard, 1981; Cherqui *et al.*, 1981; Sorge & Hilf, 1981). However, the implication of carbohydrates in the chain of subsequent events that lead to a series of biological responses is at present poorly documented. Cuatrecasas & Illiano (1971) were the first to note that sialic acid units are needed for insulin biological activity. Others have since confirmed these findings (Czech *et al.*, 1974; Solomon *et al.*, 1975; Tsuda *et al.*, 1980). A possible role for carbohydrates in insulin's action was also proposed by Katzen (1979). Our laboratory recently showed that sialylated glycosidic moieties of the complex N-linked type, containing D-galactose, D-mannose and N-acetyl-D-glucosamine units, are involved in insulin 'high-affinity' binding as well as in the activation of the membrane glucose transport system in fat-cells (Cherqui *et al.*, 1982).

The present study was designed to answer the question as to whether such surface glycosidic entities might be involved in intracellular bioactivi-

Abbreviations used: AIB, α-aminoisobutyric acid; KRB, Krebs–Ringer bicarbonate; PNA, peanut agglutinin; RCA I, *Ricinus communis* agglutinin I; SBA, soya-bean agglutinin; WGA, wheat-germ agglutinin.

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ties of insulin exerted via mechanisms that are independent of insulin effect on membrane transport processes. The experiments involved testing non-penetrating glycosidic probes on rat adipocyte metabolism of: (i) glucose, as determined by the conversion of [$3\text{-}^3\text{H}$]glucose into total ^3H -labelled lipids under conditions where glucose transport was rate-limiting (0.5 mM-glucose) or was not rate-limiting (10 mM-glucose), or completely blocked by cytochalasin B (10 mM-glucose); (ii) amino acids, assessed by α -amino[1- ^{14}C]isobutyric acid uptake, L-[U- ^{14}C]leucine incorporation into fat-cell protein and ^{14}C -labelled protein breakdown.

Our data indicate that surface sialylated glycoproteins of the complex *N*-linked type are involved in the insulin stimulation of glucose and amino acid intracellular metabolic processes. Considered together with our previous results, they suggest that both membrane-associated and intracellular effects of insulin have at least some components of their pathways of action in common, involving surface glycosylated effector entities of, or closely linked to, the insulin-receptor complex.

Materials and methods

Crude collagenase (*Clostridium histolyticum*) (EC 3.4.24.3) type I (213 units/mg) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. (lot CLS 41A264); neuraminidase (*Arthrobacter ureafaciens*) (EC 3.2.1.18) (0.5 unit/mg, where 1 unit liberates 1 μmol of sialic acid from *N*-acetylneuraminosyl-D-lactose in 1 min at 25°C and pH 7.5) from Boehringer-Mannheim (lot 1501406); concanavalin A (*Concanavalia ensiformis*), peanut (*Arachis hypogea*) agglutinin, *Ricinus communis* (castor-bean) agglutinin I, soya-bean (*Glycine max*) agglutinin, wheat-germ (*Triticum*) agglutinin and methyl α -D-mannopyranoside were purchased from Réactifs IBF, Pharmindustrie, Villeneuve-la-Garenne, France; D-galactose, *N*-acetyl-D-glucosamine, cytochalasin B, cycloheximide and bovine serum albumin (fraction V, essentially fatty acid-free) (lot 22 F-9305) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; dinonyl phthalate was from Merck, Darmstadt, Germany; ^{125}I -labelled insulin (sp. radioactivity 100 Ci/g), [$3\text{-}^3\text{H}$]glucose (sp. radioactivity 11.5 Ci/mmol), L-[U- ^{14}C]leucine (sp. radioactivity 300 Ci/mol), [^3H]inulin (sp. radioactivity 265.9 mCi/g) and Biofluor (scintillation fluid) were from New England Nuclear Corp., Boston, MA, U.S.A.; α -amino[1- ^{14}C]isobutyric acid (58 Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; pig monocomponent insulin (25 i.u./mg, where 1 i.u. is equivalent to 0.04 mg) was from Novo Research Institute, Copenhagen, Denmark. Insulin was dissolved in 0.03 M-HCl at a concentration of 1 mg/ml

and diluted in KRB buffer (119 mM-NaCl/4.8 mM-KCl / 1.2 mM - KH_2PO_4 / 1.2 mM - MgSO_4 / 1.3 mM - CaCl_2 / 25 mM- NaHCO_3), to the concentrations required in assays. All chemicals were of the highest grade available.

Lectin specificities (Goldstein & Hayes, 1978)

The lectin specificities were as follows: concanavalin A, terminal or internal α -D-mannose, α -D-glucose; PNA, terminal D-galactose, D-galactose-(β 1-3)-*N*-acetyl-D-galactosamine; RCA I, terminal or internal D-galactose; SBA, terminal α -*N*-acetyl-D-galactosamine; WGA, terminal or internal *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid.

Preparation of isolated adipocytes

Adipocytes were isolated from the epididymal fat-pads of Wistar male rats (150–175 g) by a modification of the method of Rodbell (1964) as previously described (Cherqui *et al.*, 1981). The adipocyte number was determined by counting portions of the cell suspension in a haemocytometer. Fat-cell viability was assessed by the Trypan Blue-exclusion test.

Insulin-binding studies

Fat-cells (approx. 10^5 cells) were pre-incubated at 37°C for 30 min with or without lectins (20 $\mu\text{g}/\text{ml}$) in the absence or in the presence of cytochalasin B (50 μM). The binding of ^{125}I -labelled insulin (75 pM) to fat-cells was then measured as previously described (Cherqui *et al.*, 1981). Non-specific binding (^{125}I -labelled insulin bound in the presence of 40 μg of unlabelled insulin/ml) was subtracted from the total binding to obtain the specifically bound ^{125}I -labelled insulin. Non-specific binding was never more than 15% of the total binding.

Lipogenesis

Lipogenesis was measured by the method of Moody *et al.* (1974) as the incorporation of [$3\text{-}^3\text{H}$]glucose into toluene-extractable lipid after incubation of adipocytes (approx. 10^5 cells) in KRB buffer, pH 7.4, containing defatted bovine serum albumin (10 mg/ml) and 0.5 or 10 mM-glucose. The incubation mixture (1 ml total volume) also contained 0.25 μCi of [$3\text{-}^3\text{H}$]glucose and graded concentrations of insulin or lectins. When appropriate, lectin specific monosaccharide competitors (50 mM) or cytochalasin B (50 μM) were added at the start of the incubation. The incubation vials were individually gassed with CO_2/O_2 (1:19), capped and incubated for 2 h at 37°C in a metabolic shaker at 80 strokes/min. The reaction was stopped by the addition of 15 ml of a toluene-based scintillation fluid [2,5-diphenyloxazole (5 g/litre) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.3 g/litre) in

scintillation-grade toluene] followed by vigorous shaking. After 2 h at room temperature, the radioactivity was counted in a liquid-scintillation spectrometer at a counting efficiency of about 49%. The radioactivities were corrected for quenching by external standardization. Flasks containing scintillation fluid to which the cells were added served as controls and gave a small but constant count rate, which represented non-metabolic ^3H -labelling. This control value was subtracted from basal and insulin- or lectin-stimulated rates, which were linear for at least 2 h.

cubated for an additional 2 h at 37°C either in the presence or in the absence of insulin (6 ng/ml) or lectins (40 µg/ml). The incubation buffer contained 0.1 mM-cycloheximide. At the beginning and end of the incubation period, 0.3 ml samples were centrifuged to separate cells from medium; 10% (w/v) trichloroacetic acid was then added to each fraction. Samples of the trichloroacetic acid-soluble and trichloroacetic acid-insoluble fractions were dissolved in 1 M-NaOH, added to 10 ml of Biofluor and the radioactivity was determined in a liquid-scintillation spectrometer. Protein degradation was calculated as follows:

$$\text{Amount of degradation of protein (\%)} = \frac{\text{trichloroacetic acid-soluble radioactivity in cells plus medium}}{\text{total radioactivity}} \times 100$$

Protein synthesis

Adipocytes (approx. 0.5×10^5 cells) were incubated in 0.5 ml of KRB buffer containing defatted albumin (10 mg/ml), 1 mM-glucose, 0.2 µCi of L-[U- ^{14}C]leucine and graded concentrations of insulin or lectins in the presence or absence of their respective monosaccharide competitors. The incubation vials were individually gassed with CO_2/O_2 (1:19), capped and incubated at 37°C for 2 h in a metabolic shaker at 80 strokes/min. Incubations were terminated by precipitating duplicate 0.2 ml samples with 3.8 ml of ice-cold 10% (w/v) trichloroacetic acid. After 15 min at 0°C and centrifugation for 5 min at 5000 rev./min, the precipitate was washed with 2 × 2 ml of ice-cold 10% (w/v) trichloroacetic acid, dissolved in 0.2 ml of 1 M-NaOH and assayed for protein-incorporated radioactivity in 10 ml of Biofluor. Radioactivity was determined in a liquid-scintillation spectrometer at a counting efficiency of approx. 86%. The measured radioactivities were corrected for quenching by external standardization. Control tubes contained 10% (w/v) trichloroacetic acid to which the cells were added and gave a low count rate, which represented non-metabolic ^{14}C -labelling. This control value was subtracted from basal and insulin- or lectin-stimulated rates, which were linear for at least 2 h.

Protein degradation

The degradation of ^{14}C -labelled protein was assessed by a modification of the method of Seglen *et al.* (1979). Adipocytes (approx. 1.2×10^6 cells) were incubated for 1 h at 37°C in 8 ml of KRB buffer, containing defatted albumin (10 mg/ml), 1 mM-glucose and 3.2 µCi of L-[U- ^{14}C]leucine under an atmosphere of CO_2/O_2 (1:19). Non-incorporated labelled leucine was removed, cells were resuspended in KRB buffer to a total volume of 8 ml and portions of the cell suspension (700 µl) were in-

α -Aminoisobutyric acid transport

Fat-cells (approx. 10^5 cells) were incubated at 37°C with or without graded concentrations of insulin (0.2–6 ng/ml) or lectins (2–40 µg/ml). After 2 h, transport assays were initiated by adding 50 µl of KRB buffer containing 0.25 µCi of α -amino-[1- ^{14}C]isobutyric acid ([^{14}C]AIB) and unlabelled AIB at a final concentration of 0.1 mM. AIB transport was measured at 37°C over a 10 min period (time-course experiments showed that AIB transport was linear for at least 15 min at 37°C). The assay was terminated using the oil centrifugation technique (Gliemann *et al.*, 1972), as adapted for 2-deoxyglucose transport (Cherqui *et al.*, 1982). [^3H]Inulin (1 µCi/tube), which is an extracellular marker (Gliemann *et al.*, 1972), was used for the determination of the amino acid trapped in the extracellular fluid. All results have been corrected for this factor.

Neuraminidase treatment of fat-cells

The activity and purity of the enzyme preparation were tested as described previously (Cherqui *et al.*, 1981). The absence of contaminating proteinases was confirmed: the incubation of ^{125}I -labelled insulin (0.4 ng/ml) with neuraminidase (0.05 unit/ml) for 20 min at 37°C did not decrease hormone precipitability in 5% (w/v) trichloroacetic acid. Fat-cells (approx. 10^6 cells) were incubated at 37°C for 20 min in the absence or in the presence of neuraminidase (0.05 unit/ml). The reaction was stopped by washing fat-cells with 3 × 5 ml of KRB buffer, pH 7.4, containing defatted albumin (10 mg/ml). Cell viability was verified by the Trypan Blue-exclusion test and their lipid and protein syntheses tested.

Statistical analysis

Results are given as means $\pm$ S.E.M. for the

indicated numbers of observations (n) made with separate preparations of fat-cells. Differences between the mean values were evaluated by Student's t -test.

Results

Comparative effects of insulin and lectins on lipogenesis from [3-³H]glucose

In an attempt to dissociate the effects of insulin and lectins on the intracellular metabolism of glucose from those on membrane glucose transport (Cherqui *et al.*, 1982), lipogenesis from [3-³H]glucose was compared in control and insulin- and lectin-treated fat-cells at two glucose concentrations.

At 0.5 mM-glucose, a concentration at which glucose transport is considered as a rate-limiting step for subsequent glucose metabolism, insulin and lectins produced a dose-dependent increase in basal lipogenesis (Fig. 1). The stimulatory effect of insulin was maximal at 0.13 nM (0.8 ng/ml) and resulted in about a 3-fold increase in the basal level (Fig. 1a); half-maximal stimulation (ED_{50}) occurred at a concentration of 0.023 nM-insulin, in agreement with results reported previously (Katzen *et al.*, 1981). When fat-cells were incubated with increasing concentrations (1–40 μ g/ml) of either concanavalin A (Fig. 1b), WGA (Fig. 1c) or RCA I (Fig. 1d), maximal effects were observed at a lectin concentration of 20 μ g/ml and were respectively 87, 81 or 82% of that elicited by insulin. The ED_{50} values were respectively 4.5, 1 and 1.5 μ g/ml for concanavalin A, WGA and RCA I. Maximal lectin and insulin effects were not additive; concanavalin A (Fig. 1b), WGA (Fig. 1c) and RCA I (Fig. 1d), all at 20 μ g/ml, did not enhance basal lipogenesis beyond the maximal effect obtained with insulin. The stimulatory effect of concanavalin A, WGA and RCA I on lipogenesis was completely abolished in the presence of the respective monosaccharide competitors (50 mM) (Figs. 1b–1d), indicating that this was related to the lectins' sugar specificities.

At 10 mM-glucose, glucose transport is no longer a rate-limiting step for glucose metabolism (Crofford & Renold, 1965). In this situation, the incubation of fat-cells with insulin (0.1–6 ng/ml) or lectins (1–40 μ g/ml) still produced a dose-dependent increase in basal lipogenesis; maximal activation (1.6–1.8-fold) was observed at 0.8 ng of insulin/ml or 20 μ g of concanavalin A, WGA or RCA I/ml (Fig. 2a). The persistent stimulation of lipogenesis at 10 mM-glucose by either insulin or lectins suggested that both agents exert direct intracellular effects on glucose metabolism. This point was further supported by experiments performed in the presence of cytochalasin B at a concentration (50 μ M) that completely abolishes facilitated transport in fat-cells (Czech *et al.*, 1973). Fig. 2(b) shows that the metabolism of

[3-³H]glucose (which had entered the cells by simple diffusion) fell to 47% of the control value without cytochalasin B and was still maximally increased by 1.5–1.8-fold with both insulin and lectins at concentrations of 0.8 ng/ml and 20 μ g/ml respectively. It was moreover observed that, whether or not cytochalasin B was present in the incubation medium: (i) insulin and lectin maximal effects were not additive (Fig. 2); (ii) concanavalin A, WGA and RCA I maximal stimulatory effects were decreased by about 56, 67 and 60% in the presence of the respective monosaccharide competitors (50 mM) ($n = 3$). This finding reflected a decreased ability of specific sugars to antagonize lectin effects at 10 mM-compared with at 0.5 mM-glucose.

The possibility that cytochalasin B might affect the interaction of either insulin or lectins with insulin receptors was ruled out by the results presented in Table 1. Cytochalasin B (50 μ M) failed to alter the binding of 75 pM-¹²⁵I-labelled insulin (a concentration producing a submaximal stimulation of lipogenesis) or the lectin-induced inhibition of this process; concanavalin A, WGA and RCA I (20 μ g/ml) decreased insulin binding by about 50, 56 and 37% respectively, whether or not cytochalasin B was present.

To gain further insight into a possible relationship between lectin stimulatory effects on lipogenesis and the abilities of lectins to decrease insulin binding, the effects of two other lectins, peanut and soya-bean agglutinins, known not to have any effect on the insulin–receptor interaction (Cherqui *et al.*, 1981; Katzen *et al.*, 1981; Shechter & Sela, 1981), were studied. Neither of them significantly modified lipogenesis from [3-³H]glucose (117 $\pm$ 10 or 112 $\pm$ 12% of the control value at 0.5 mM-glucose and 109 $\pm$ 5 or 105 $\pm$ 7% of the control value at 10 mM-glucose for PNA and SBA respectively) ($n = 2$).

Comparative effects of insulin and lectins on L-[U-¹⁴C]leucine incorporation into fat-cell protein

Fig. 3 compares the dose–response curves for the effects of insulin, concanavalin A and WGA on L-[U-¹⁴C]leucine incorporation into fat-cell protein. Insulin was most effective at 1 nM (6 ng/ml) and led to a 1.5-fold increase in the ¹⁴C-labelled protein content of fat-cells (Fig. 3a). The ED_{50} was 0.063 nM, in agreement with previous results (Mine-mura *et al.*, 1970). When fat-cells were incubated with increasing concentrations (2–100 μ g/ml) of either concanavalin A (Fig. 3b) or WGA (Fig. 3c), maximal activation occurred at a lectin concentration of 40 μ g/ml and was equivalent to that elicited by a maximally effective concentration of insulin. The ED_{50} values were 4 and 1.5 μ g/ml for concanavalin A and WGA respectively. The combined effects of insulin and lectins at maximally

Fig. 1. Comparative effects of insulin and lectins on lipogenesis from $[3-^3\text{H}]$ glucose (0.5 mM)

Fat-cells (approx. 10^5 cells) were incubated for 2 h at 37°C with $0.25 \mu\text{Ci}$ of $[3-^3\text{H}]$ glucose at a final concentration of 0.5 mM and graded concentrations of: (a), insulin; (b), (c) and (d), concanavalin A, WGA or RCA I respectively in the absence (continuous lines) or in the presence (broken lines) of their respective monosaccharide competitors (methyl α -D-mannopyranoside, *N*-acetyl-D-glucosamine or D-galactose, all at 50 mM); in some experiments, fat-cells were incubated in the simultaneous presence of 0.8 ng of insulin/ml and 20 μg of concanavalin A, WGA or RCA I/ml (open symbols in b, c and d respectively). Total extractable lipids were determined as described in the Materials and methods section. The amount of $[3-^3\text{H}]$ glucose converted into total ^3H -labelled lipids for the control (100%) was $11.65 \pm 1.74 \text{ nmol/2h}$ per 10^5 cells. Each point represents the mean $\pm$ s.e.m. for duplicate determinations from three (broken lines and open symbols) to seven (a) or nine (continuous lines in b, c and d) separate experiments. Values significantly different from the control value are indicated: * $P < 0.02$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 2. Comparative effects of insulin and lectins on lipogenesis from $[3\text{-}^3\text{H}]$ glucose (10 mM)

Fat-cells (approx. 10^5 cells) were incubated for 2 h at 37°C with $0.25\text{ }\mu\text{Ci}$ of $[3\text{-}^3\text{H}]$ glucose at a final concentration of 10 mM and with the indicated concentration of insulin (hatched columns), and without insulin (open column, 'basal'), or with lectins alone (open columns) and plus 0.8 ng of insulin/ml (hatched columns). Incubations were performed in the absence (a) or in the presence (b) of cytochalasin B ($50\text{ }\mu\text{M}$). Total extractable lipids were determined as described in the Materials and methods section. Data are means $\pm$ S.E.M. for duplicate determinations from the indicated numbers of separate experiments (numbers in parentheses). Values significantly different from the control values are indicated: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$. Abbreviation used: ConA, concanavalin A.

effective concentrations did not surpass the maximal effect achieved by either agent alone, thus indicating their non-additivity (Figs. 3b and 3c). Whatever the lectin concentrations used, concanavalin A and WGA stimulatory effects were almost completely abolished in the presence of the respective mono-saccharide competitors (50 mM) (Figs. 3b and 3c); this finding argued for lectins' specific effects.

In contrast with the results observed for lipogenesis, RCA I ($2\text{--}40\text{ }\mu\text{g/ml}$) produced a dose-dependent decrease in the incorporation of $\text{L-[U-}^{14}\text{C]leucine}$ into cellular protein: a 48% inhibition was observed at $40\text{ }\mu\text{g/ml}$ (Table 2). The RCA I-A chain has recently been shown to be toxic to 80S ribosomes in plant or animal translation systems (Harley & Beevers, 1982). This property probably accounts for our observation.

In order to determine whether insulin, concanavalin A and WGA increased the ^{14}C -labelled protein content of fat-cells by activating amino acid transport across the membrane, comparisons were made of their effects on $[^{14}\text{C}]$ AIB uptake. Insulin

($0.2\text{--}6\text{ ng/ml}$), concanavalin A or WGA ($2\text{--}40\text{ }\mu\text{g/ml}$) failed to enhance AIB uptake in fat-cells under the conditions of incubation (2 h at 37°C) at which they fully stimulated $\text{L-[U-}^{14}\text{C]leucine}$ incorporation into cellular protein; the amounts of AIB transported were respectively 13.85 ± 1.16 , 12.88 ± 1.41 , 11.08 ± 1.30 and $12.16 \pm 1.18\text{ pmol/min per } 10^5$ cells for control and insulin-, concanavalin A- and WGA-treated fat-cells ($n = 6$).

Concentrations of insulin and lectins shown to be maximally effective on protein synthesis did not significantly modify the degradation of cellular ^{14}C -labelled protein. Under these conditions, the amounts of ^{14}C -labelled protein degraded were 5.02 ± 0.24 , 5.12 ± 0.23 , 5.47 ± 0.50 and $5.00 \pm 0.20\%$ /2h respectively for control and insulin-, concanavalin A- and WGA-treated fat-cells ($n = 3$).

Neither PNA nor SBA ($2\text{--}100\text{ }\mu\text{g/ml}$) significantly modified $\text{L-[U-}^{14}\text{C]leucine}$ incorporation into fat-cell protein (95 ± 3 and $91 \pm 4\%$ of the control value respectively) ($n = 2$).

Table 1. Lack of effect of cytochalasin B on the interaction of insulin or lectins with fat-cell insulin receptors
Fat-cells (approx. 10⁵ cells) were incubated for 30 min at 37°C with or without the indicated concentrations of lectins either in the absence or in the presence of cytochalasin B (50 μM). ¹²⁵I-labelled insulin-binding studies were then conducted as described in the Materials and methods section. Values are means ± s.e.m. for quadruplicate determinations from three separate experiments. Numbers in parentheses indicate the results expressed as percentages of the control value. Results significantly different from the control value are indicated: *P < 0.05; **P < 0.02; ***P < 0.01.

Additions	Specific ¹²⁵ I-labelled insulin binding (fmol/10 ⁵ cells)	
	Without cytochalasin B	With 50 μM-cytochalasin B
None	0.419 ± 0.045 (100)	0.395 ± 0.039 (94)
Concanavalin A (20 μg/ml)	0.198 ± 0.024** (47)	0.218 ± 0.047* (52)
WGA (20 μg/ml)	0.182 ± 0.011*** (43)	0.190 ± 0.013*** (45)
RCA I (20 μg/ml)	0.249 ± 0.042* (59)	0.280 ± 0.024* (67)

Effect of neuraminidase on insulin and lectin stimulation of lipogenesis and protein synthesis

To gain further insight into the intracellular insulin-like activities of lectins on lipogenesis and protein synthesis, the effects of insulin and lectins on both processes were compared in fat-cells pre-incubated for 20 min at 37°C in the presence or in the absence of neuraminidase (0.05 unit/ml). The viability of the treated or untreated fat-cells at the end of the incubation was greater than 95% (as estimated by the Trypan Blue-exclusion test).

Basal lipogenesis (10 mM-glucose) and protein synthesis were not significantly modified in the neuraminidase-treated fat-cells compared with the untreated controls (see the legend to Table 2); nor was passive diffusion of 0.25 mM-2-deoxyglucose (non-metabolizable sugar analogue) altered, as evaluated by measurement of sugar uptake in the presence of 50 μM-cytochalasin B (results not shown). These findings indicate that cellular integrity is preserved in the neuraminidase-treated fat-cells.

When, however, fat-cells were pre-incubated with neuraminidase, neither the maximally effective concentrations of insulin nor of lectins stimulated lipid or protein synthesis (Table 2).

Fig. 3. Comparative effects of insulin and lectins on the incorporation of L-[U-¹⁴C]leucine into fat-cell protein
Fat-cells (approx. 0.5 × 10⁵ cells) were incubated for 2 h at 37°C with 0.2 μCi of L-[U-¹⁴C]leucine in a final volume of 0.5 ml and graded concentrations of: (a), insulin; (b) and (c), concanavalin A and WGA respectively in the absence (continuous lines) or in the presence (broken lines) of their respective monosaccharide competitors (methyl α-D-mannopyranoside or N-acetyl-D-glucosamine, both at 50 mM); in some experiments, fat-cells were incubated in the simultaneous presence of 6 ng of insulin/ml and 40 μg of concanavalin A or WGA/ml (open symbols in b and c respectively). The amount of L-[U-¹⁴C]leucine incorporated into fat-cell protein for the control (100%) was 3.64 ± 0.21 pmol/2h per 10⁵ cells. Each point represents the mean ± s.e.m. for quadruplicate determinations from three (broken lines and open symbols in b and c), six (a) or seven (continuous lines in b and c) separate experiments. Values significantly different from the control value are indicated: *P < 0.05; **P < 0.01; ***P < 0.001.

Table 2. *Effect of neuraminidase on insulin and lectin stimulation of lipid and protein syntheses*

Fat cells (approx. 10^6 cells) were incubated for 20 min at 37°C in the absence or in the presence of neuraminidase (0.05 unit/ml). At this time, fat-cells were washed with 3×5 ml of KRB buffer, pH 7.4, containing defatted bovine serum albumin (10 mg/ml). Assays for the conversion of $[3\text{-}^3\text{H}]\text{glucose}$ (10 mM) into ^3H -labelled lipids and the incorporation of $\text{L-}[U\text{-}^{14}\text{C}]\text{leucine}$ into fat-cell protein were then conducted as described in the Materials and methods section, either in the absence or in the presence of insulin or lectins. The designated quantities are per 0.5 ml (protein synthesis) or per 1 ml (lipogenesis). The control values of $[3\text{-}^3\text{H}]\text{glucose}$ converted into total ^3H -labelled lipids were 20.40 ± 2.44 and 23.20 ± 3.48 nmol/2 h per 10^5 cells for non-treated and neuraminidase-treated fat-cells respectively. The control values of $\text{L-}[U\text{-}^{14}\text{C}]\text{leucine}$ incorporated into fat-cell protein were 5.38 ± 0.60 and 6.38 ± 0.95 pmol/2 h per 10^5 cells for non-treated and neuraminidase-treated fat-cells respectively. Values are means $\pm$ S.E.M. for quadruplicate (protein synthesis) or duplicate (lipogenesis) determinations from three separate experiments. Values significantly different from the control values are indicated: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$.

Additions	[3- ^3H]Glucose converted into total ^3H -labelled lipids (% of control)		L-[U- ^{14}C]Leucine incorporated into fat-cell protein (% of control)	
	Non-treated fat-cells	Neuraminidase-treated fat-cells	Non-treated fat-cells	Neuraminidase-treated fat-cells
None (control)	100	100	100	100
Insulin (ng)				
0.8	151 $\pm$ 9*	94 $\pm$ 5		
3.0			147 $\pm$ 8**	102 $\pm$ 4
Concanavalin A (μg)				
20.0	158 $\pm$ 10*	99 $\pm$ 4	143 $\pm$ 10*	87 $\pm$ 9
WGA (μg)				
20.0	173 $\pm$ 9***	103 $\pm$ 13	145 $\pm$ 11*	92 $\pm$ 9
RCA I (μg)				
20.0	168 $\pm$ 12**	87 $\pm$ 3	52 $\pm$ 7*	

Discussion

Specific non-penetrating glycosidic probes were used to investigate the involvement of sialylated glycoproteins of the complex *N*-linked type in the insulin stimulation of glucose and amino acid intracellular metabolic processes.

The methodological problems related to the conditions used for fat-cell isolation, enzyme or lectin treatment have been described in detail previously (Cherqui *et al.*, 1981). The present discussion only deals with the specificity of lectin effects and the choice of appropriate substrates for studying lipid and protein syntheses. (1) Lectin stimulatory effects on lipogenesis and protein synthesis were markedly, if not totally, inhibited in the presence of the respective monosaccharide competitors, providing evidence for a specific interaction of lectins with cell-surface carbohydrate determinants. (2) $[3\text{-}^3\text{H}]\text{Glucose}$ was shown to be a particularly suitable substrate for lipid conversion (Moody *et al.*, 1974). This property was attributed to the ability of ^3H on C-3 of glucose to be transferred mainly to water and NADP^+ , the resultant $[^3\text{H}]\text{NADP}^+$ being used for reductive steps in fatty acid synthesis. Furthermore, $\text{L-}[U\text{-}^{14}\text{C}]\text{leucine}$ incorporation into fat-cell protein was shown to be not only more efficient but also to be more stimulated by insulin than other amino acids (Minemura *et al.*, 1970).

The comparative effects of insulin and lectins on fat-cell lipogenesis were studied at two glucose concentrations. With 0.5 mM-glucose, a concentration at which glucose transport is thought to be a rate-limiting step for glucose metabolism, the concanavalin A, WGA and RCA I stimulatory effects on lipogenesis were chiefly attributable to the ability of lectins to mimic the effect of insulin on glucose transport (Cherqui *et al.*, 1982). As insulin and lectins still stimulated significantly lipid synthesis under conditions where glucose transport was either no longer rate-limiting (10 mM-glucose) or completely abolished (by 50 μM -cytochalasin B), they were clearly able to enhance the intracellular metabolism of glucose via mechanisms additional to that for the activation of glucose transport across the membrane. This property of insulin has also been demonstrated in other studies using different methodological approaches (Olefsky, 1976; Garratt *et al.*, 1979). Several arguments indicate that lectins faithfully reproduce insulin's intracellular effects. (i) Stimulatory effects of both insulin and lectins on lipogenesis at 10 mM-glucose were lower than those observed at 0.5 mM, whether or not cytochalasin B was present in the incubation medium. This observation is consistent with the decrease in the insulin stimulation of glucose metabolism as glucose concentrations are raised and confirms previous

findings (Crofford, 1968; Olefsky, 1976). (ii) Lectin stimulatory effects could not be due to an increase in the passive diffusion of glucose, since this process was shown not to be affected by either concanavalin A, WGA or RCA I (Cherqui *et al.*, 1982). (iii) Both insulin and lectin stimulatory effects observed in the presence of cytochalasin B were due to post-receptor rather than to receptor events, since this agent failed to significantly modify either insulin binding or the lectin-induced decreases of this process. Thus, while a substantial part of the concanavalin A, WGA and RCA I effects on lipogenesis appeared to be related to stimulation of glucose transport, intracellular insulin-like effects of lectins were evident as well. The fact that concanavalin A was able to reproduce the effect of insulin on the activities of either pyruvate dehydrogenase in a subcellular system (Seals & Jarett, 1980) or glycogen synthase in the absence of glucose (Lawrence & Larner, 1978) further substantiates the present findings.

In order to characterize carefully the concanavalin A and WGA insulin-like effects on amino acid metabolism, which, to our knowledge, have not been described before, an attempt was made to determine whether the primary site of action of the lectins was at the level of amino acid transport, protein synthesis or protein breakdown. Our results show that insulin effects on amino acid metabolism, which fully agreed with previous findings (Goodman, 1966; Clausen & Rodbell, 1969; Minemura *et al.*, 1970), were faithfully mimicked by concanavalin A and WGA. Thus, both insulin and lectins failed to change significantly amino acid transport or protein breakdown; both were equally effective stimulators of L-[U-¹⁴C]-leucine incorporation into fat-cell protein. These findings indicated that intracellular protein synthesis is the process that is stimulated by either insulin or lectins. Further evidence in support of this hypothesis comes from the identification of peptide chain initiation as a site of insulin's action on protein synthesis in rat adipocytes (Lyons *et al.*, 1980).

The above data indicate that lectins stimulate glucose and amino acid intracellular metabolic processes under conditions where they have no effect upon membrane transport systems. They moreover provide supportive evidence for a common pathway for both insulin and lectin effects as well as for both insulin bioactivities as follows. (i) Concanavalin A, WGA and RCA I insulin-like effects on glucose and amino acid intracellular metabolic processes were related to the ability of lectins to inhibit insulin binding. PNA and SBA, which did not alter insulin binding (Cherqui *et al.*, 1981), were ineffective in stimulating either lipid or protein synthesis. Such a correlation has been reported previously (Katzen *et al.*, 1981) and suggests that the insulin-like activities of lectins are mediated through lectin interaction with insulin receptors. (ii) Lectin and insulin maxi-

mal stimulatory effects on lipid and protein syntheses were not additive, indicating a common effector system for both lectin and insulin bioactivities on either of the processes studied. (iii) Lectin and insulin effects on either glucose or amino acid intracellular metabolic processes were both abolished by neuraminidase treatment of fat-cells; this finding indicated that sialylated glycosidic moieties are implicated in lectin and insulin bioeffects and added further support to the concept of a common glycosylated effector system for both lectins and insulin. It moreover suggested that the effects of insulin on these two entirely different processes are mediated in a co-ordinated manner through a common mechanism or a common initial event.

From these data, it is concluded that surface sialylated glycoproteins of the complex N-linked type are involved in the insulin stimulation of glucose and amino acid intracellular metabolic processes. Such glycosylated entities have already been shown to be involved in insulin 'high affinity' binding and activation of glucose transport (Cherqui *et al.*, 1982). Thus, the methodological approach developed in the present study, with specific non-penetrating and/or multivalent glycosidic probes as complementary tools, provided two important insights into the mechanism of insulin action. The first is that several biological responses can be initiated by the interaction of ligands other than insulin with the insulin receptor; this further substantiates the concept that the receptor contains all the necessary information for insulin bioactivity (Kahn *et al.*, 1978; Van Obberghen *et al.*, 1979). Our results indicate that the binding of insulin to glycosylated receptor subunits is likely to generate some common type of trans-membrane signal via effector entities on the cell surface that are also glycosylated. Whether these entities are a part of the insulin-receptor complex and may, therefore, be identified as the β -subunits (Kasuga *et al.*, 1982), or are closely linked to the receptor, remains to be elucidated. The second major point is that aggregation or cross-linking of insulin receptors appear to be important in insulin action, as previously reported (Kahn *et al.*, 1978). Larner *et al.* (1981) have proposed that these processes probably initiate the proteolytic events that occur at the external cell surface and lead to oligo-glycopeptide mediator formation.

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References

- Capeau, J. & Picard, J. (1980) *FEBS Lett.* **118**, 25–29
- Capeau, J., Picard, J. & Caron, M. (1978) *Cancer Res.* **38**, 3930–3937
- Caron, M. & Picard, J. (1981) *Biochim. Biophys. Acta* **648**, 137–144
- Caron, M., Picard, J. & Kern, P. (1978) *Biochim. Biophys. Acta* **512**, 29–40
- Cherqui, G., Caron, M., Capeau, J. & Picard, J. (1981) *Mol. Cell. Endocrinol.* **23**, 297–310
- Cherqui, G., Caron, M., Capeau, J. & Picard, J. (1982) *Mol. Cell. Endocrinol.* **28**, 627–643
- Clausen, T. & Rodbell, M. (1969) *J. Biol. Chem.* **244**, 1258–1262
- Crofford, O. B. (1968) *J. Biol. Chem.* **243**, 362–369
- Crofford, O. B. & Renold, A. E. (1965) *J. Biol. Chem.* **240**, 14–21
- Cuatrecasas, P. (1971) in *Insulin Action* (Fritz, I. B., ed.), pp. 137–171, Academic Press, New York
- Cuatrecasas, P. (1973) *J. Biol. Chem.* **248**, 3528–3534
- Cuatrecasas, P. & Illiano, G. (1971) *J. Biol. Chem.* **246**, 4938–4946
- Czech, M. P., Lynn, D. G. & Lynn, W. S. (1973) *J. Biol. Chem.* **248**, 3636–3641
- Czech, M. P., Lawrence, J. C. & Lynn, W. S. (1974) *J. Biol. Chem.* **249**, 7499–7505
- Garratt, C. J., Brown, D. & Evans, R. M. (1979) *Int. J. Biochem.* **10**, 943–945
- Gliemann, J., Osterlind, K., Vinten, J. & Gammeltoft, S. (1972) *Biochim. Biophys. Acta* **286**, 1–9
- Goldstein, I. J. & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem.* **35**, 127–340
- Goodman, H. M. (1966) *Am. J. Physiol.* **211**, 815–820
- Harley, S. M. & Beavers, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5935–5938
- Hedo, J. A., Kasuga, M., Van Obberghen, E., Roth, J. & Kahn, C. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4791–4795
- Herzberg, V., Boughter, J. M., Carlisle, S. & Hill, D. E. (1980) *Nature (London)* **286**, 279–281
- Kahn, C. R., Baird, K. L., Jarrett, D. B. & Flier, J. S. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4209–4213
- Kasuga, M., Hedro, J. A., Yamada, K. M. & Kahn, C. R. (1982) *J. Biol. Chem.* **257**, 10392–10399
- Katzen, H. M. (1979) *J. Biol. Chem.* **254**, 2983–2992
- Katzen, H. M., Vicario, P. P., Mumford, R. A. & Green, B. G. (1981) *Biochemistry* **20**, 5800–5809
- Larner, J., Cheng, K., Schwartz, C., Dubler, R., Creacy, S., Kikuchi, K., Tamura, S., Galasko, G., Pullin, C. & Katz, M. (1981) *Mol. Cell. Biochem.* **40**, 155–161
- Lawrence, J. C. & Larner, J. (1978) *J. Biol. Chem.* **253**, 2104–2113
- Lyons, R. T., Nordeen, S. K. & Young, D. A. (1980) *J. Biol. Chem.* **255**, 6330–6334
- Massague, J., Pilch, P. F. & Czech, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7137–7141
- Minemura, T., Lacy, W. W. & Crofford, O. B. (1970) *J. Biol. Chem.* **245**, 3872–3881
- Moody, A. J., Stan, M. A. & Stan, M. (1974) *Horm. Metab. Res.* **6**, 12–16
- Olefsky, J. M. (1976) *Biochem. Biophys. Res. Commun.* **71**, 106–113
- Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–400
- Sandra, A., Leon, M. A. & Przybylsky, R. J. (1979) *Endocrinology* **105**, 391–401
- Seals, J. R. & Jarrett, L. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 77–81
- Seglen, P. O., Grinde, B. & Solheim, A. E. (1979) *Eur. J. Biochem.* **95**, 215–225
- Shechter, Y. & Sela, B. A. (1981) *Biochem. Biophys. Res. Commun.* **98**, 367–373
- Solomon, S. S., King, L. E. & Hashimoto, K. (1975) *Horm. Metab. Res.* **7**, 297–304
- Sorge, L. K. & Hilf, R. (1981) *Biochim. Biophys. Acta* **676**, 187–198
- Tsuda, M., Taketomi, S. & Iwatsuka, H. (1980) *Am. J. Physiol.* **239**, E186–E191
- Van Obberghen, E., Spooner, P. M., Kahn, C. R., Chernick, S. S., Garrison, M. M., Karlsson, K. A. & Grunfeld, C. (1979) *Nature (London)* **280**, 500–502