A Postbinding Inhibitor of Insulin Action

Increased Concentrations in the Plasma of Non-insulin-dependent Diabetic Subjects

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SUMMARY

"Postreceptor" insulin resistance in persons with non-insulin-dependent diabetes (NIDDM) could be due to an intrinsic defect in insulin-sensitive pathways or to the action of a circulating inhibitor. Since evidence for the former is lacking, we have addressed the question of a circulating inhibitor by examining the effect of plasma and plasma extracts from NIDDM subjects on the lipogenic response of rat adipocytes to insulin.

A majority (77%) of plasma samples (1:20 dilution) from unselected, treated NIDDM subjects (N = 69) inhibited insulin-stimulated conversion of 3-^{3}H-glucose to ^{3}H-lipid in rat adipocytes to a greater extent than did control samples (N = 24). The mean ± SD inhibition by NIDDM plasma (81 ± 21%) was significantly greater ($P < 0.01$) than by control plasma (50 ± 14%). Diabetic and, to a lesser degree, control plasma both caused a significant decrease in the maximal response of lipogenesis to insulin. Inhibitory activity was extracted into acid/ethanol, present in the flow of a Sep-pak C18 column, heat-stable (56°C for 30 min [plasma], 80°C for 30 min [acid/ethanol]), resistant to proteases, and dialyzable through 1000-dalton-mol wt exclusion dialysis tubing. The inhibition by NIDDM plasma or partially purified inhibitor could not be explained by the presence of insulin antibodies, insulin receptor antibodies, other inhibitors of insulin binding, or the concentrations of known counterregulatory factors. There was no correlation between inhibitory activity and plasma glucose (r = 0.26), insulin (r = 0.33), C-peptide (r = 0.26), or HbA_{1c} (r = 0.26).

Our findings suggest that a humoral inhibitor of postreceptor insulin action is present in increased concentrations in the plasma of NIDDM subjects. The nature of this inhibitory activity and its pathophysiologic significance deserve further investigation. DIABETES *33*:450–454, May 1984.

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Received for publication 24 May 1983 and in revised form 9 September 1983.

Insulin resistance, a feature of both nonobese and obese subjects with non-insulin-dependent diabetes mellitus (NIDDM),[1] is mainly attributed to a postbinding defect in insulin action.[2] Such a defect could be intrinsic or mediated by a humoral factor, being either genetic ("inborn") or acquired. Attempts to demonstrate an intrinsic postbinding defect in insulin action using cultured human fibroblasts from NIDDM subjects have been unrewarded.[3,4] Before the advent of the insulin radioimmunoassay, it was fashionable to ascribe NIDDM to a circulating inhibitor of insulin action.[5] Inhibitory activity has, in fact, been demonstrated in the sera of rats with experimental insulin-dependent diabetes,[6–8] and two studies[9,10] provide evidence for inhibitory activity in human sera. One early study[9] demonstrated an inhibition of insulin-stimulated glycogen synthesis in the isolated rat hemidiaphragm by sera from seven subjects with "insulin resistant diabetes"; inhibitory activity was not present in "non-resistant diabetic" and normal sera. A later study[10] reported the isolation of an insulin antagonist from the sera of both NIDDM and insulin-dependent subjects, thought to be of pituitary origin. Recently, we reported the presence of a circulating inhibitor of postbinding, insulin action in the plasma of a young female with insulin resistance and acanthosis nigricans.[11] This finding, and the previous reports of insulin-inhibitory activity in NIDDM sera, led us to reexamine the question of circulating inhibitors of insulin action and their role in NIDDM. We report, initially, the inhibitory effects of plasma and plasma extracts from NIDDM subjects on the lipogenic response of rat adipocytes to insulin, and the partial purification of an inhibitory factor present in plasma.

MATERIALS AND METHODS

Subjects. Heparinized blood was obtained, with consent, from randomly selected fasting subjects with NIDDM attending the clinics of The Royal Melbourne Hospital, and from normal volunteers. No attempt was made to classify patients according to sex, age, weight, or treatment. Glu-

cose, hemoglobin A_{1c}, immunoreactive insulin, and C-peptide were measured on the plasma samples. All samples were screened for the presence of insulin antibodies,[12] insulin receptor antibodies by immunoprecipitation of solubilized human placental receptors,[13] and for their effect on stimulation of lipogenesis by insulin in isolated rat adipocytes (see below).

Forty diabetic plasmas shown to inhibit insulin-stimulated lipogenesis were pooled, as were the 20 control plasmas. These plasma pools were used in attempts to isolate and characterize the inhibitory activity. Insulin, somatomedin-C, and the counterregulatory hormones (growth hormone, glucagon, cortisol, and prolactin) were measured in the pools by standard radioimmunoassays. Binding and degradation of ^{125}I-insulin by adipocytes were measured in the presence of pooled plasma (see below).

Lipogenesis assay. The lipogenesis assay was modified after Moody et al.;[14] the use of glucose labeled in the C-3 position (3-^{3}H-glucose) ensured the maximal incorporation of label via NADPH into fatty acids, as opposed to glycerol.[14] Epididymal fat pads from 120–150-g male rats were digested in 3 mg/ml collagenase (Worthington Crude B, 131 U/ml) for 45 min at 37°C (1.5 ml/rat) in Dulbecco's buffer (NaCl 7.36 g/L, KCl 0.2 g/L, $CaCl_2$ 0.1 g/L, Na_2HPO_4 1.15 g/L, KH_2PO_4 0.2 g/L, and $MgCl_2$, $6H_2O$ 0.21 g/L), pH 7.4, containing 1 mmol/L glucose and 4% bovine serum albumin (Sigma RIA grade). Liberated adipocytes, separated from remaining intact tissue by rinsing through a sieve, were washed three times by flotation in assay buffer (Dulbecco's containing 1 mmol/L glucose and 1% bovine serum albumin), resuspended, and incubated at 37°C for 1 h with gentle shaking before use. Fat pads from one animal provided sufficient adipocytes for 15 assay tubes. Reactions were performed in polyethylene tubes containing adipocytes, 0.1 μCi 3-^{3}H-glucose (Amersham), 50 μl of plasma without (basal) or with (stimulated) 4 ng of insulin in a final volume of 1.0 ml of assay buffer. After 2-h incubation at 37°C, the conversion of 3-^{3}H-glucose to ^{3}H-lipid was measured as the radioactivity extracted into a toluene-based scintillant. The 1:20 dilution of plasma in the assay was shown to be the highest dilution of normal plasma that consistently gave maximal inhibition of insulin-stimulated lipogenesis, although some diabetic plasmas still inhibited maximally at lower dilutions.

To allow comparison of results from different assays, the following formula was used: percent inhibition by plasma = 100 − [(plasma stimulated − plasma basal)/(buffer stimulated − buffer basal) × 100].

Studies with pooled plasma. The lipogenic response to a range of insulin concentrations (0–40 ng/ml) was measured in the presence of pooled diabetic or control plasmas.

Dialysis experiments were carried out in an attempt to define the molecular weight of the inhibitor. Pooled diabetic plasma was dialyzed against 500 vol of Dulbecco's assay buffer, overnight, in 1000- or 10,000-dalton-mol wt exclusion dialysis tubing, and tested for an effect on lipogenesis.

To purify the inhibitory activity, pooled plasma was initially extracted into acid/ethanol. One milliliter of pooled plasma was added to 9 ml of acid/ethanol (10 ml distilled water, 200 ml 1 M hydrochloric acid, and 790 ml ethanol), mixed at 4°C overnight, and centrifuged at 3000 rpm for 15 min. The supernatant was adjusted to pH 7.4, freeze-dried, and reconstituted to the original plasma volume. Aliquots of the acid/ethanol extract were assayed for an effect on lipogenesis as described for plasma. In addition, the acid/ethanol extract was diluted 1:4 with acidified 0.9% NaCl (pH 3.0) and applied to a Waters C18 Sep-pak column. After washing with 5 ml acidified NaCl, the column was eluted with increasing concentrations of acetonitrile. The flow-through fraction and eluate fractions were freeze-dried, reconstituted to the original extract volume, adjusted to pH 7.4, and assayed for activity in the lipogenesis assay.

To determine whether insulin was degraded or its binding impaired in the presence of plasma, ^{125}I-insulin (20,000 cpm; 100 μCi/μg) was incubated with adipocytes in 1 ml of Dulbecco's assay buffer containing either 50 μl of diabetic or control pooled plasma, in the absence or presence of 1 μg of unlabeled insulin. After 15 min at 37°C, the assay medium was separated from the cells by centrifugation through dinonylphthalate oil. Radioactivity bound to adipocytes was counted in a gamma spectrometer and specific binding determined by subtracting the radioactivity bound in the presence of excess unlabeled insulin. The integrity of unbound ^{125}I-insulin was assessed by measuring the percent radioactivity in the separated medium precipitated by 5% trichloroacetic acid (TCA). In a parallel experiment, basal and insulin-stimulated lipogenesis, in the presence of diabetic or normal pooled plasma, was measured at 15 min to demonstrate inhibition at this time point.

RESULTS

Several plasmas containing insulin antibodies were excluded from further study, as their binding of insulin in the assay system would have led to inhibition of insulin action. Insulin receptor antibodies were not detected in any plasma sample.

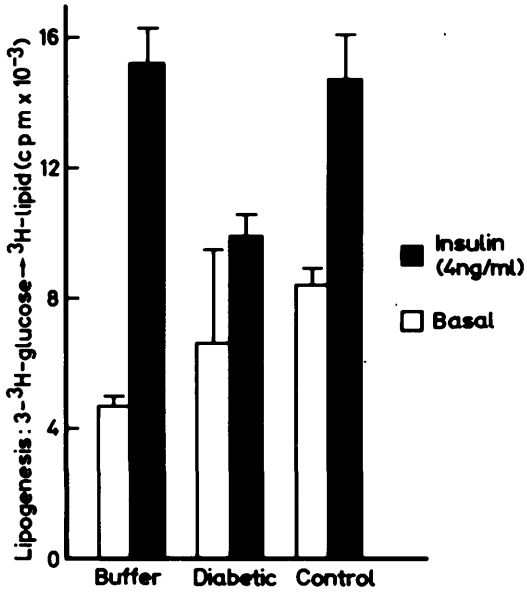

FIGURE 1. Lipogenesis assay (nonstandardized data): mean ± 2 SD of 3-^{3}H-glucose to ^{3}H-lipid by 1:20 dilution of diabetic plasma (N = 5) and normal control plasma (N = 8) in the absence (basal) or presence (stimulated) of exogenous insulin.

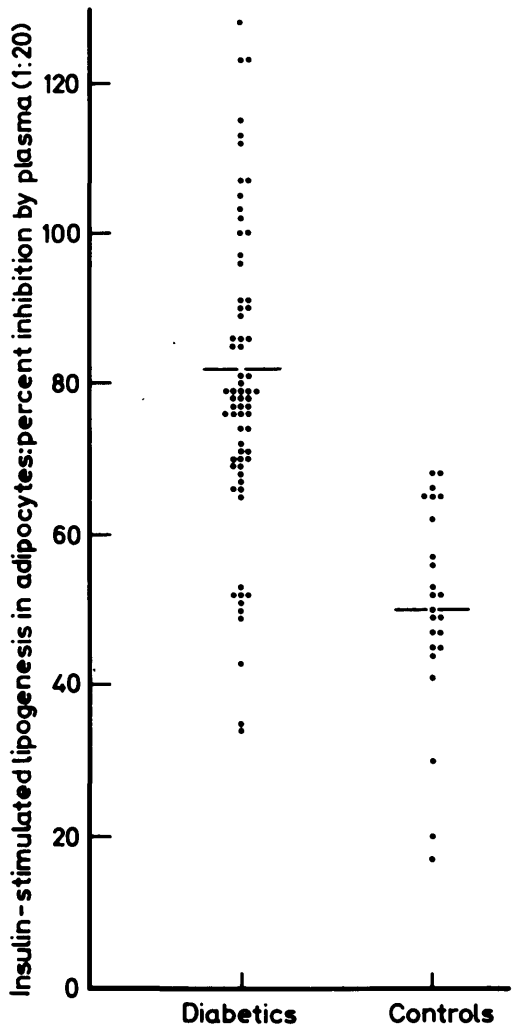

FIGURE 2. Inhibition of insulin-stimulated lipogenesis (standardized data) by 1:20 dilution of diabetic plasma or normal control plasmas.

Effect of plasma on insulin-stimulated lipogenesis. The data from a typical assay (Figure 1) show that NIDDM plasmas significantly decrease the lipogenic response to insulin when compared with control plasmas, or to buffer alone. The increase in basal lipogenesis over buffer alone, in the presence of either diabetic or control plasma, cannot be attributed to the concentration of endogenous insulin, and presumably reflects the insulin-like activity, especially NSILA-p,[15] of whole plasma. Despite this increase in basal activity, the response to insulin in the presence of control plasma was not increased above buffer alone; in the presence of NIDDM plasma it was significantly reduced.

Interassay variation in the lipogenic activity of fat cells necessitated standardization of the responses (see MATERIALS AND METHODS). Thus, of 69 plasma samples from patients with NIDDM, 53 (77%) showed a greater inhibition of insulin-stimulated lipogenesis than did control plasmas (Figure 2). The mean ± SD inhibitory activity of NIDDM plasma was 81 ± 21% compared with 50 ± 14% for the control plasma ($P < 0.01$).

There was no correlation between inhibitory activity and the plasma glucose ($r = 0.26$), insulin ($r = 0.33$), C-peptide ($r = 0.26$), or HbA_{1c} ($r = 0.26$) levels.

Studies with pooled plasma. There were no significant differences in the concentrations of the various counterregulatory hormones measured in the pooled diabetic and control plasmas.

Maximum insulin-stimulated lipogenesis was markedly decreased in the presence of pooled diabetic plasma, with a less significant decrease in the presence of pooled control plasma (Figure 3). Inhibition by plasma appeared to be present at all insulin concentrations, although this effect was obscured at low concentrations of insulin by the insulin-like activity of plasma. The double-reciprocal plot of this data indicated that the inhibition of insulin action was of a noncompetitive nature. Specific ^{125}I-insulin binding to adipocytes at 15 min was identical in the presence of pooled diabetic plasma (2.8%) and pooled control plasma (2.9%). Before incubation, ^{125}I-insulin was 96% precipitable by TCA; after incubation with adipocytes and diabetic plasma or control plasma, TCA precipitability at 15 min was 81% and 80%, respectively. Increased inhibition of lipogenesis by pooled diabetic plasma, compared with pooled normal plasma or buffer control, was apparent after 15 min of incubation (data not shown).

Inhibitory activity was absent after dialyzing plasma across 1000- and 10,000-dalton "cut-off" membranes (Figure 4). The small increase in the basal insulin-like activity of the dialysate may suggest that inhibition of non-insulin-stimulated lipogenesis may also have been lost. Loss of inhibition after dialysis could not be accounted for by binding of the "inhibitor" to the dialysis membrane, since inhibitory activity was unaltered in plasma left in contact with open dialysis membrane overnight (data not shown).

Inhibitory activity was shown to be present in the acid/ethanol extract of both diabetic and control pooled plasma (Figure 5). The marked decrease in basal lipogenesis can

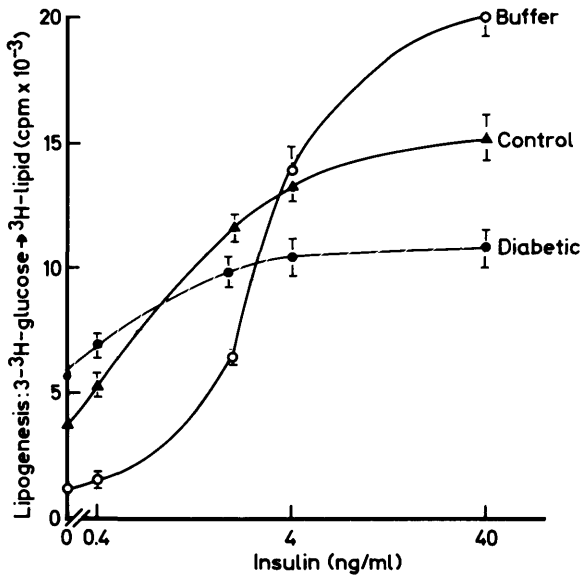

FIGURE 3. Insulin stimulation curve: stimulation of lipogenesis by exogenous insulin (mean ± 2 SD) in the presence of a 1:20 dilution of diabetic plasma or normal control plasma.

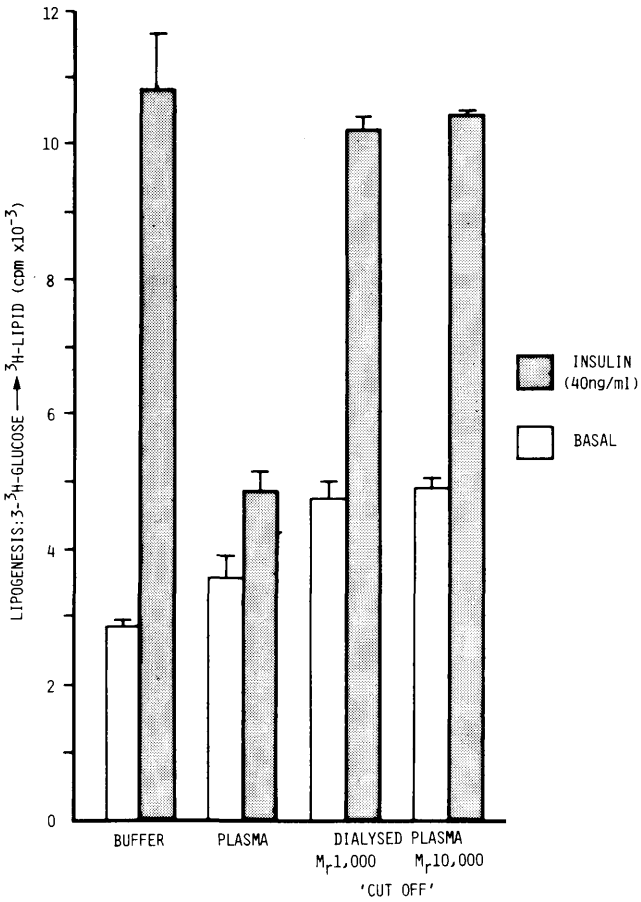

FIGURE 4. Inhibition of insulin-stimulated lipogenesis by plasma dialysates after dialysis across 1000- and 10,000-dalton "cut off" membranes against 500 vol of buffer.

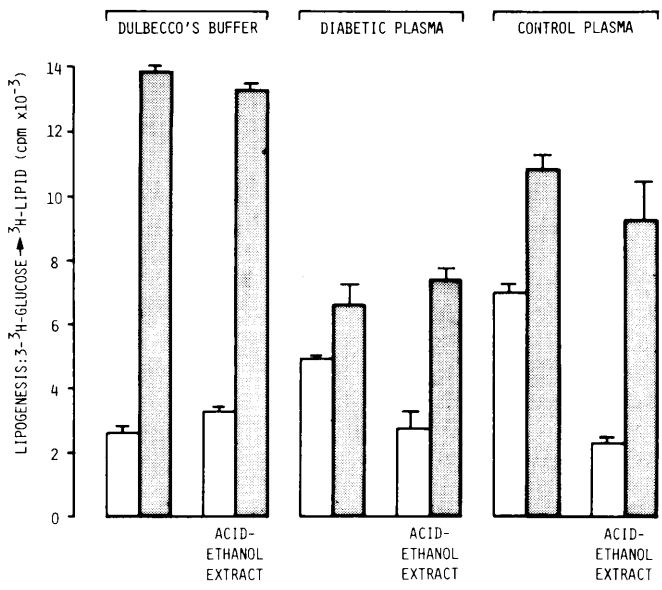

FIGURE 5. Effect of acid/ethanol extract of diabetic or normal pooled plasma on insulin-stimulated lipogenesis.

be accounted for by the removal of the NSILA-p (precipitated) fraction of the plasma, which is thought to contribute 80% of the insulin-like activity of plasma.[15]

Inhibitory activity was present in the flow-through fraction from the C18 Sep-pak column and in decreased concentrations in the NaCl column wash, but was absent in all of the fractions eluted with acetonitrile (Figure 6). Cortisol, growth hormone, glucagon, and catecholamines were absent from the Sep-pak flow-through fraction, and free fatty acid, cholesterol, and triglyceride levels were reduced to < 10% of the original pool-plasma concentration (data not shown).

Plasma-inhibitory activity was shown to be heat-stable (56°C for 30 min) as was the inhibitory activity extracted in acid/ethanol (80°C for 30 min). Plasma-inhibitory activity was also shown to be resistant to treatment with trypsin and pronase (500 μg/ml, 60 min, 37°C) (data not shown).

DISCUSSION

We have demonstrated that plasma inhibits insulin-stimulated lipogenesis in the rat adipocyte, with plasma from NIDDM subjects being significantly more potent than that from control subjects. The inhibitory activity of plasma was not associated with a decrease in insulin binding, while the increased inhibition by NIDDM plasma or plasma extracts could not be accounted for by increased insulin degradation or levels of known counterregulatory hormones, free fatty acids, or lipids. The present data indicate that the inhibitor is of low molecular weight, is acid-stable, heat-stable, and resistant to proteases; the fact that it does not bind to the Sep-pak C18 column indicates that it is likely to be extremely hydrophilic.

Whether diabetic plasma simply contains more inhibitory activity than is present in control plasma, or whether there is a qualitative difference between the two, is uncertain. However, our findings suggest that in both cases inhibition is occurring at a postbinding step in insulin-sensitive pathways, resulting in a decreased maximal response to insulin. The lipogenesis assay does not allow us to define the locus of the inhibitory effect. However, preliminary studies have

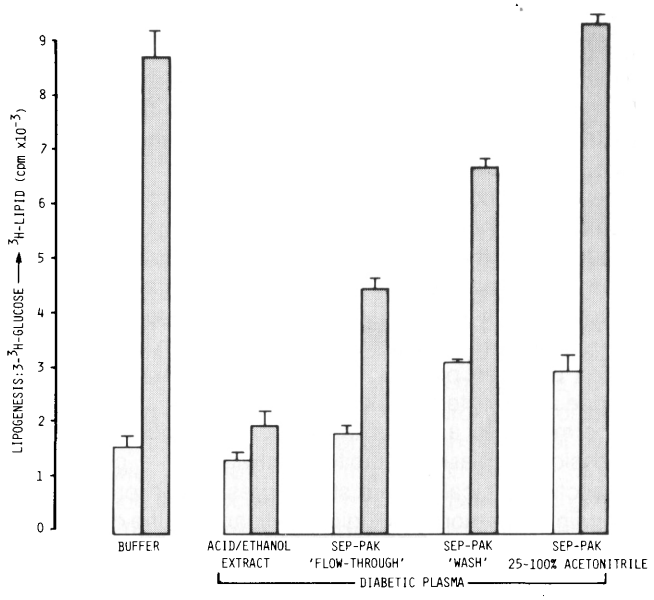

FIGURE 6. Sep-pak C18 chromatography: lipogenic response to insulin in the presence of acid/ethanol plasma fractions after reverse-phase chromatography.

shown that diabetic plasma also has a significant inhibitory effect on 2-deoxy-glucose transport in rat adipocytes, suggesting that the inhibition of lipogenesis is secondary to impaired glucose utilization.

We are aware of only one prior report of NIDDM plasma inhibiting insulin action more than normal plasma or plasma from insulin-dependent diabetic subjects.[9] However, in that study inhibition of insulin-stimulated glycogen synthesis in isolated rat hemidiaphragms was overcome by increasing the concentration of insulin, indicating, in contrast to the present findings, an effect on sensitivity rather than on maximum responsiveness. Other workers[9] claim that a growth hormone-derived peptide isolated from sera inhibits insulin action via an effect on glyceraldehyde-3-phosphate dehydrogenase, α-glycerophosphate dehydrogenase, and acetyl-CoA-carboxylase; the greatest inhibitory effect was obtained with plasma from insulin-dependent subjects or NIDDM subjects on insulin therapy.[10] Our data suggest that the inhibitory activity demonstrated in NIDDM subjects is not due to a large peptide and, furthermore, we have been unable to show increased inhibitory activity in the plasma of five insulin-dependent subjects (unpublished observations). It therefore seems unlikely that we are reporting on the same phenomenon.

Humoral inhibition of insulin action has been reported in other situations. A circulating inhibitor of insulin-stimulated glycogen synthesis in the isolated rat hemidiaphragm was proposed as a cause of insulin resistance associated with diabetic acidosis.[16] The inhibitory activity was associated with the α-globulin fraction of serum, was not simply due to a low serum pH, was not a lipoprotein, did not compete with ^{131}I-insulin for binding sites on the rat hemidiaphragm, and disappeared on patient recovery. Another study reported inhibitory activity on insulin-stimulated glucose utilization by isolated rat hemidiaphragms in plasma from insulin-resistant subjects with hepatic cirrhosis;[17] however, these workers could not demonstrate inhibitory activity in plasma from patients with NIDDM. Postreceptor defects causing insulin resistance have been demonstrated in a number of other disease states, including renal failure,[18] in which a humoral inhibitor might logically be implicated. Before attaching significance to the various circulating "insulin antagonists," careful characterization of their chemical and functional properties will be required.

The lack of correlation between the inhibitory activity in plasma and other parameters (fasting glucose, insulin, C-peptide, and HbA_{1c} levels) in the diabetic subjects is not surprising, given our semi-quantitation of inhibitory activity in whole plasma and the fact that subjects were unselected. However, in the index patient we have shown a direct correlation between plasma inhibitory activity and insulin resistance as reflected by plasma insulin levels.[11] Our data do not permit us to address the influence of obesity on the expression of plasma inhibitory activity, but a controlled, prospective clinical patient study, presently in progress to ascertain the pathophysiologic significance of the circulating inhibitor, will hopefully clarify this point.

Our findings lend weight to the hypothesis that defective glucose utilization through insulin-sensitive pathways in some subjects with NIDDM is due to a postbinding humoral inhibitor rather than to an intrinsic or genetic defect in the cell. Firm evidence in favor of a pathophysiologic role for a circulating inhibitor(s) of insulin action will require further purification and quantitation.

ACKNOWLEDGMENTS

We wish to thank Drs. F. I. R. Martin, J. Proietto, P. Colman, and C. Ley for assistance with the collection of blood samples; Dr. D. Campbell for measuring glucose, catecholamines, and HbA_{1c}; Dr. T. Billington for measurement of free fatty acids; and Linda Stafford for excellent secretarial assistance. This work was supported in part by a research grant from The Clive and Vera Ramaciotti Foundation.

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