In Vitro Control of T-Lymphocyte Insulin Receptors by In Vivo Modulation of Insulin

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SUMMARY

The mechanism for carbohydrate intolerance related to insulin resistance is under intense study. Some have suggested that environmental modulation of plasma insulin concentrations may influence cellular mechanisms that could result in diminished receptor binding and thus create insulin resistance. We have employed the T-lymphocyte model to explore this possibility. Two groups of studies were performed. In the first, relative hyperinsulinemia was created in patients with type I diabetes mellitus by means of continuous subcutaneous insulin infusion by a portable insulin infusion device. In response to an elevation of "free" plasma insulin from 14 ± 2 to 26 ± 3 μU/ml there was a progressive fall in the number of insulin receptors that developed on freshly isolated, then lectin-treated, cultured T-lymphocytes (6552 ± 377 to 3241 ± 348 sites/cell) after 14 days of hyperinsulinemia. Neither the level of lectin stimulation, as ascertained by ^{3}H-thymidine uptake, nor the time course of response to PHA was altered by the insulin therapy. In the second study, plasma insulin was reduced in normal-weight subjects and in nondiabetic obese subjects (nearly twice ideal body weight) by means of a 4-day fast. In response to a fall in plasma insulin of 35 ± 6 to 19 ± 3 μU/ml, insulin receptor number on freshly isolated, then cultured and stimulated, lymphocytes rose from 2796 ± 379 sites/cell to 6453 ± 893 sites/cell. Again, neither a change in lectin responsiveness nor a change in the time course of PHA response accounted for the change in these data. The relationships between the direction and magnitude of changes in in vivo plasma insulin levels in both studies were analyzed together. There was a strong ($r = 0.94$; $P < 0.001$) inverse relationship between changes in plasma insulin levels and the ultimate number of insulin receptors displayed on cultured lymphocytes. We conclude that in vivo insulin modulation is important for the regulation of the appearance of insulin receptors on cultured T-lymphocytes. Since the T-cell does not bear an insulin receptor in the circulation, the regulatory signals cannot be passed to this cell by the binding event itself. These data are not explained by a shift in a subset of lymphocytes present or by alterations in the degree of cell activation induced by PHA. The T-lymphocyte capacity to synthesize insulin receptors appears to be set, in part, by ambient plasma insulin concentrations. DIABETES *32*:712–717, August 1983.

Based on examination of the insulin receptor present on multiple tissues, it has been suggested that environmental factors may influence cellular mechanisms that could result in disorders of carbohydrate metabolism. The concept is that some examples of carbohydrate intolerance due to insulin resistance are caused by defective insulin receptor binding, which is a consequence of environmental modulation of the concentration of the ligand itself.[1] Our laboratory has been interested in the insulin receptor that develops on the T-lymphocyte after cellular activation. This cell has several useful features that make it a relevant model for study of intrinsic receptor characteristics. The T-lymphocyte in the circulation is generally devoid of insulin receptors. If the cell is removed from the circulation and challenged with a mitogen in tissue culture, an insulin receptor emerges upon the membrane as early as 24 h later.[2-4] Thus, the insulin receptor is generated on a cell in tissue culture under controlled conditions. We recently demonstrated a diminished insulin receptor number in static T-cell binding studies of mitogen-stimulated T-cells obtained from obese, nondiabetic subjects and from patients with type II diabetes mellitus.[5] We offered two possible explanations for these results. One explanation was that T-cells from such subjects may be genetically preprogrammed to develop a diminished number of insulin binding sites on the cell surface after stimulation. Thus, the number of insulin receptor sites on the surface of this cell, and perhaps on

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TABLE 1
Clinical characteristics of patients with type I diabetes mellitus

Patient	Age (yr)	Sex	Height (cm)	Weight (kg)	%IBW	Duration (yr)	Insulin dosage (U/day) Conventional Rx	Insulin dosage (U/day) CSII Rx	Plasma "free" insulin (μU/ml) Conventional Rx	Plasma "free" insulin (μU/ml) CSII Rx	Glucose CSII Rx (mg/dl) Day 0	Glucose CSII Rx (mg/dl) Day 4	Glucose CSII Rx (mg/dl) Day 14
1	42	F	170.0	67.5	1.06	30	41	48	19	32	262	182	48
2	31	M	166.4	60.9	1.01	28	80	44	21	30	319	116	64
3	20	F	170.2	63.7	1.07	4	80	44	19	32	183	66	102
4	27	M	174.2	68.6	1.04	13	40	29	10	13	245	76	66
5	35	F	158.7	50.2	0.98	19	30	31	9	30	210	119	81
6	25	F	160.4	56.9	1.08	16	30	27	10	17	101	95	57
7	22	F	150.8	47.9	1.01	10	34	37	12	29	217	100	101
Mean ± SEM	29 ± 3		164.4 ± 3	59.4 ± 3	1.04 ± 0.01	17 ± 4	40 ± 7	37 ± 3	14 ± 2	26 ± 3	220 ± 26	108 ± 14	74 ± 8

other cells, may be genetically determined and not be the consequence of environmental changes in circulating insulin levels. An alternative suggestion was that the T-lymphocyte synthetic capacity for the insulin receptor was set by the ambient circulating insulin concentration to which it was previously exposed. Corollary to the latter thought is that the T-cells from such patients, as a result of exposure to a hyperinsulinemic environment, will generate fewer insulin receptors. The T-lymphocyte is subject to well-described modes of insulin receptor regulation operative in other cell types with the unique difference being that the consequence of regulation is observed ex vivo, in tissue culture.

The purpose of the studies described in this paper was to explore the regulation of the T-cell insulin receptor by in vivo alterations in the ambient plasma insulin concentration. The results of these studies strongly suggest that the development of the insulin receptor ex vivo is set by circulating plasma levels in vivo.

MATERIALS AND METHODS

The effect of relative hyperinsulinemia on the development of the T-lymphocyte insulin receptor. In order to study the effect of relative hyperinsulinemia on the regulation of the T-cell insulin receptor, seven normal-weight patients with type I diabetes mellitus were studied. These patients all had typical type I diabetes and, except for their diabetes, were otherwise well, all being free of significant microvascular complications of their disease. The clinical characteristics of these patients are detailed in Table 1. All were admitted to the General Clinical Research Unit, Parkland Memorial Hospital, for a 3-wk protocol of investigation, the details of which have been described previously.[6] In brief, they were placed on a weight-maintaining diet consisting of 50% carbohydrate, 20% protein, and 30% fat. On the third hospital day, the initial receptor studies were performed while the patients received their usual dose of conventionally administered insulin. In most cases this was two injections of a mixture of intermediate- and short-acting insulin. After completion of the initial studies, patients were treated with a continuous subcutaneous insulin infusion (CSII) using a portable insulin infusion device. Our protocol for this therapy delivered via the infusion device was chosen to provide normalization of plasma glucose concentration and to create relative hyperinsulinemia. Repeat receptor studies were performed on day 4 and day 14 after creation of hyperinsulinemia by CSII. On the morning of each study (0800), after an overnight fast, blood was obtained for measurement of plasma glucose and free insulin and for analysis of T-lymphocyte insulin receptors.

TABLE 2
Clinical characteristics of subjects participating in the 96-h fast

Patient	Age (yr)	Sex	Height (cm)	Weight (kg)	%IBW	Plasma insulin (μU/day) Pre fast	Plasma insulin (μU/day) Post fast	Serum glucose (mg/dl) Pre fast	Serum glucose (mg/dl) Post fast
Normal									
1	43	F	165.1	54.0	0.95	8	4	93	60
2	25	M	184.2	80.4	1.08	12	7	105	61
3	20	M	181.6	66.8	0.88	12	7	80	70
4	30	F	165.1	54.7	0.96	13	14	78	56
5	24	M	178.8	79.0	1.03	18	6	90	80
6	25	M	172.5	67.5	0.98	10	3	90	63
Mean ± SEM	28 ± 3		174.6 ± 3	67.1 ± 5	0.98 ± 0.03	12 ± 1	7 ± 2	89 ± 4	65 ± 4
Obese									
1	32	M	180.9	123.4	1.58	16	10	92	90
2	37	F	152.4	108.3	2.38	29	19	87	78
3	21	F	166.4	104.0	1.79	44	16	82	74
4	36	F	171.5	129.5	2.07	53	27	85	57
5	30	F	162.6	94.0	1.72	31	21	94	67
Mean ± SEM	31 ± 3		166.7 ± 5	111.8 ± 6	1.91 ± 0.14	35 ± 6	19 ± 3	88 ± 2	73 ± 6

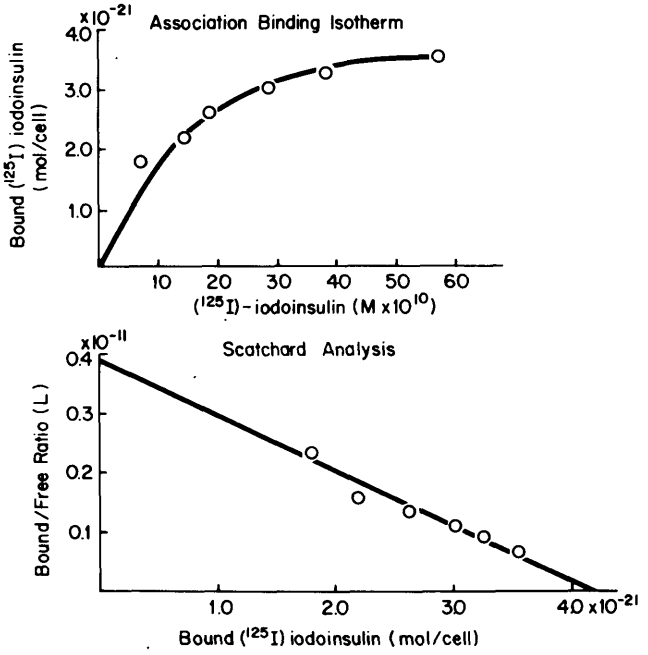

FIGURE 1. Radiolabeled insulin binding to T-lymphocytes recovered from a patient with type I diabetes mellitus after 14 days of CSII. The upper panel displays the association binding isotherm. The lower panel demonstrates the Scatchard transformation of the data. The least-squares fit of this set of data gives an equation of the line: y = $-(0.096 \times 10^{10}\ 1/M)X + 0.393 \times 10^{-11}$ L, with a regression coefficient = 0.955. The inverse of the slope provides the affinity of the receptor for insulin, here 1.04 nM. The X intercept gives the V_{max} of binding in mol/cell, which when multiplied by Avogadro's number will provide the number of receptors per cell, here 2466 sites/cell.

The effect of reducing plasma insulin levels on the development of the T-lymphocyte insulin receptor. To examine T-lymphocyte insulin receptor regulation during reduction in hyperinsulinemia, five obese (nearly twice ideal body weight), nondiabetic subjects and six normal-weight, nondiabetic subjects were studied. The clinical characteristics of these subjects are displayed in Table 2. All subjects were admitted to the General Clinical Research Unit and fasted for 96 h. Only calorie-free liquids were permitted. To insure adequacy of fast, patients were weighed daily. All exhibited steady weight loss; urinary ketones were present in all subjects 48 h after initiation of the fast. Blood samples for plasma glucose and insulin and for analysis of T-lymphocyte insulin receptors were obtained before and at the completion of the 4-day fast.

Cell preparation. Details of the cell preparation technique have been reported previously.[5] In short, heparinized blood was diluted 1:3 in Hanks Salt Solution (HBSS). Mononuclear cells were recovered from the interface of a ficoll-Hypaque density gradient. T-cell-enriched cell populations were recovered from the effluent of a nylon wool column. Details of the degree of T-cell enrichment and cell surface marker analysis may be found in previous communications.[2,5] Macrophages, which bear insulin receptors when freshly isolated from peripheral blood, were thus virtually excluded. These T-cells (>90%) were then planted in tissue culture in RPMI 1640 buffered with 10 mM HEPES and enriched with 10% insulin-poor (<1.5 μU/ml) fetal calf serum and with 3.75 μg/ml phytohemagglutinin-P, a lectin we have shown readily initiates synthesis of the T-cell insulin receptor.[3,4] Culture was effected for 48 h in a humidified atmosphere of 5% CO_2 and air after which the activated T-cells were recovered and placed at 10^7/ml in HBSS–0.1% bovine serum albumin for the measurement of the insulin receptor.

Insulin binding assay. The basic assay technique, a modification of that described by Gammeltoft and Gliemann,[7,8] has been described previously.[2,9] Previous communications have detailed the nature of non-specific binding, the form of the Scatchard relationship, success of separation of bound from free radiolabeled insulin, and the pharmacokinetic characteristics of the T-cell insulin receptor.[2,5,9] For these studies enough cells from each subject were lectin treated to permit detection of equilibrium binding characteristics gleaned from analysis of association binding isotherms. Increasing concentrations of [^{125}I]-iodoinsulin (0–60 ng/ml) were presented to cells with and without excess (4 μg/ml) unlabeled insulin. Since the T-cell system gives a linear Scatchard,[2,5] receptor characteristics were determined from analysis of the resultant Scatchard relationships in which the X intercept gives the binding capacity and, when multiplied by Avogadro's constant, the number of receptors per cell. The negative of 1/slope gives the affinity. At least six binding points were available for each subject. For acceptability in these studies a least-squares fit of the individual Scatchard data was com-

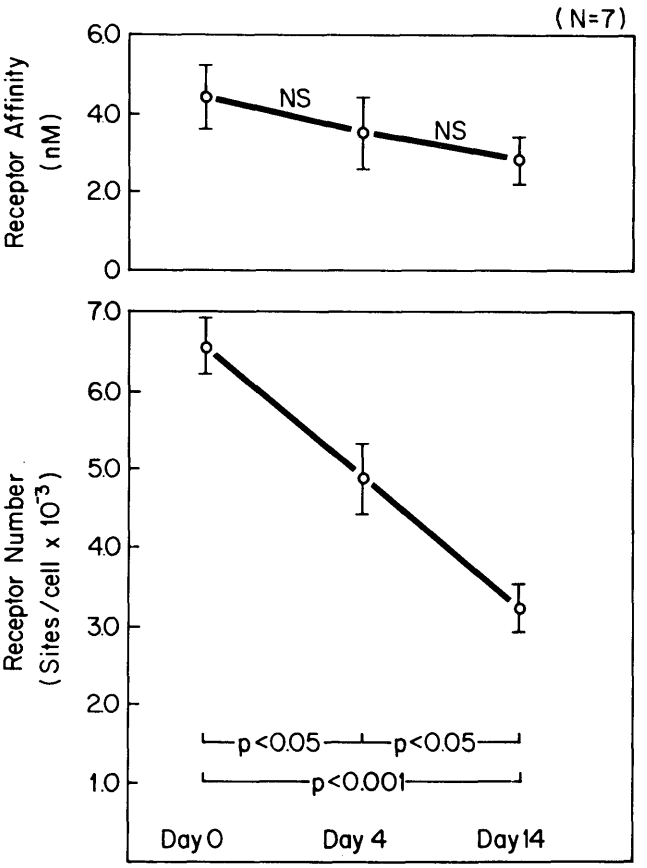

FIGURE 2. The effect of relative hyperinsulinemia on lectin-stimulated T-lymphocyte insulin receptor binding. Seven patients with type I diabetes mellitus were assayed before and after 4 and 14 days of CSII. Mean receptor affinity obtained from individual Scatchard plots is displayed in the upper panel. The number of insulin receptor sites per T-cell is displayed in the lower panel.

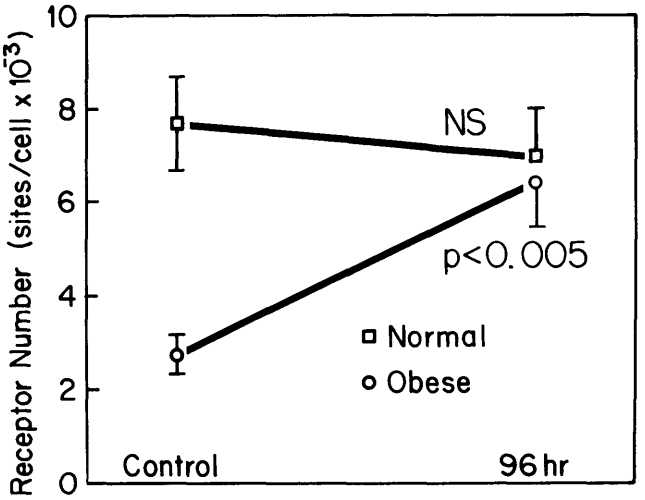

FIGURE 3. The effect of plasma insulin reduction during a 4-day total fast in normal individuals and in obese, nondiabetic subjects on lectin-stimulated T-lymphocyte insulin receptor number.

puted. Only when the fit exhibited a statistically significant coefficient of regression and an r value of ≥0.7 was the equation of the linear relationship used to provide receptor characteristics.

Measurement of plasma glucose and immunoreactive insulin. The plasma glucose concentration was measured using a glucose-oxidase system on a Beckman glucose analyzer (Beckman Instruments, Fullerton, California). Insulin was measured by the Herbert modification[10] of the assay originally described by Yalow and Berson.[11] In the patients with type I diabetes who had circulating antibodies to insulin in their plasma, "free insulin" levels were measured after treatment of the plasma with polyethylene glycol.[12]

RESULTS

The effect of relative hyperinsulinemia on the development of the T-lymphocyte insulin receptor. The goal to create relative hyperinsulinemia by CSII was achieved as plasma "free" insulin rose from 14 ± 2 to 16 ± 3 μU/ml ($P < 0.001$, paired t) after 14 days of therapy. A representative association binding isotherm of radiolabeled insulin to the T-cell insulin receptor and its linear Scatchard transformation to illustrate the manner in which receptor analysis was performed for each subject at each time period is shown in Figure 1. In response to modest, sustained hyperinsulinemia freshly isolated, then lectin-treated, cultured T-cells demonstrated no significant change in affinity of the insulin receptor for its ligand at the fourth or fourteenth day of pump therapy (Figure 2). In contrast there was a progressive fall in the number of receptors that appeared on cultured T-cells when the cells were isolated at the fourth and fourteenth day of sustained mild hyperinsulinemia from a basal number of 6552 ± 377 sites/cell, comparable to our previous reported value in this group,[5] to 4881 ± 519 sites/cell at 4 days and then to 3241 ± 348 sites/cell after 14 days of hyperinsulinemia. The effect of hyperinsulinemia in modulating the receptor number of freshly isolated, then lectin-treated T-cells was evident in each of the seven subjects studied (Figure 2). In order to determine whether hyperinsulinemia altered lectin responsiveness, ^{3}H-thymidine uptake was measured concomitant with each binding study. There were no significant differences in this estimate of DNA synthesis when cells were removed from any given patient on any day of study of the pump protocol and altered with the lectin. Moreover, there was no difference in the time course of ^{3}H-thymidine response in the pump-treated patients. Thus, one can conclude from this study that insulin receptor negative T-lymphocytes isolated from type I diabetic patients experiencing modest chronic hyperinsulinemia display fewer receptors after lectin stimulation in tissue culture than they do in the absence of hyperinsulinemia before removal of the cells.

The effect of reducing plasma insulin levels on the development of the T-lymphocyte insulin receptor. The goal to reduce plasma insulin by fasting was satisfied in both normal-weight and obese groups (Table 2). In the normal-weight subjects, immunoreactive insulin concentration fell from 12 ± 1 to 7 ± 2 μU/ml ($P < 0.05$, paired t), while plasma insulin fell from the hyperinsulinemic level of 35 ± 6 to 19 ± 3 μU/ml in the obese subjects ($P < 0.02$). The effect of reduction of plasma insulin levels on freshly isolated, then lectin-treated T-lymphocytes was disparate in the two groups. In the nonobese, nondiabetic subjects, receptor display on cultured T-cells was unaffected by reducing plasma insulin concentration within the normal range (Figure 3). In these six subjects, we found 7806 ± 1052 sites/cell before fasting and 7147 ± 1176 after 96 h of fasting. In contrast a reduction in plasma insulin levels from hyperinsulinemic concentrations toward normal in obese, nondiabetic subjects resulted in a substantial rise in the number of insulin receptors on lectin-activated T-cells approaching a normal complement (Figure 4). Insulin receptor number increased from 2796 ± 379 sites/cell, a result similar to that we have pre-

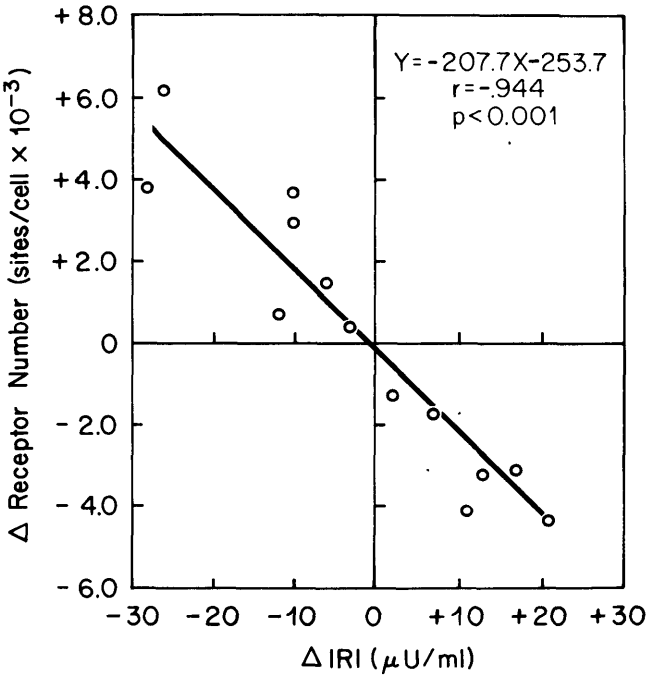

FIGURE 4. The relationship between the degree of change in immunoreactive insulin during both fasting and infusion protocols and the degree of change in lectin-stimulated T-lymphocyte insulin receptor number. This plot is constructed from all data points available on the type I diabetic patients and the obese patients.

viously reported in this group,[5] to 6453 ± 893 after the 96-h fast ($P < 0.005$, paired t). Again the effect of insulin reduction on alteration of insulin receptor number on T-cells could not be explained by the magnitude of lectin responsiveness. We conclude from this study that T-cells removed from obese subjects with basal hyperinsulinemia display a reduced number of insulin receptors on T-cells freshly isolated, then stimulated in tissue, then cultured as compared with T-cells isolated from normal-weight subjects who have normal plasma insulin concentrations. Moreover, reduction in plasma insulin concentration from hyperinsulinemic levels toward normal provides a signal to the circulating T-cell such that the cell is capable of expressing a more normal complement of insulin receptors when later activated in culture. That reduction of plasma insulin levels from a normal concentration in the nonobese subjects was not associated with alterations in lectin-generated receptor number suggests that there may be a threshold set of the insulin-sensing mechanism of the T-lymphocyte.

The relationship between changes in ambient plasma insulin levels and T-lymphocyte insulin receptor number. The relationship between the direction and magnitude of changes created in in vivo plasma insulin levels and the changes in T-lymphocyte insulin receptor number was explained by analysis of the data from both studies taken together (Figure 4). There is a strong ($r = 0.94$) and statistically significant ($P < 0.001$) inverse relationship between changes in insulin level and ultimate receptor number found in highly varied subjects undergoing two disparate experimental protocols.

DISCUSSION

Insulin binding to its receptor is not a fixed process, but is regulated by genetic and/or environmental factors. The elucidation of these factors may be helpful in furthering our understanding of the pathophysiology of diabetes and in the creation of improved treatment strategies. Insulin receptor display can be regulated with respect to the absolute number of such molecules present on a cell surface, to the strength of binding between the receptor and insulin (affinity), or both. The mechanism by which such fine tuning of the receptor-insulin system is accomplished remains speculative and controversial. Investigators using traditional target tissues for the study of the biologic activity of insulin have described the primacy of environmental factors for receptor regulation, the most important of which appears to be the ambient concentration of insulin itself.[13,14] An inverse relationship appears to exist between the concentration of plasma insulin and the number of cell receptors.[1,14–17] One hypothesis advanced to explain receptor regulation by the insulin molecule involves the binding by the ligand followed by internalization of the receptor-hormone complex, reducing the number of sites at the membrane for subsequent binding events.[18] Most of the studies that support this hypothesis use tissues that have insulin receptors present on their surface at all times. The use of those tissues might make it difficult to identify alternate regulatory mechanisms that do not require insulin binding to the receptor as the initial step. In order to address the issue of the important regulatory role played by the insulin receptor, we have turned to a model, the T-lymphocyte, which differs from others in that the cell type studied does not generally bear an insulin receptor while the cell is circulating in vivo.[2,19,20] Placed into tissue culture with an activating stimulus such as a mitogen, an insulin receptor appears on the T-lymphocyte surface within 48 h. The conditions under which the receptor appears and is regulated, then, are entirely under experimental control.

In earlier studies, our laboratory has described reduced T-lymphocyte insulin receptor number on cells isolated from patients with type II diabetes mellitus or normoglycemic, obese subjects and then stimulated in tissue culture.[5] Two interpretations of these data obtained in static, unperturbed conditions were that the reduced receptor number reflected a genetically programmed defect or that T-cells had the capacity to detect changes in the ambient plasma insulin concentration despite the fact that they had no insulin receptor in vivo. The studies reported here directly tested the second interpretation by examining the result of alterations in in vivo plasma insulin concentration on the subsequent development of insulin receptors on T-lymphocytes isolated during the perturbation and then stimulated in tissue culture. The inverse relationship between the direction of the change in plasma insulin concentration in vivo and the subsequent number of insulin receptors that appeared on the T-cells after lectin stimulation strongly supports this second view.

The two protocols used in this study to perturb in vivo plasma insulin alter the metabolic set of the patient in a complex way. Extensive fasting and insulin infusion will alter plasma glucose, free fatty acids, glucagon levels in the least, as well as insulin. The changes in insulin receptor display observed in this study may be related in part to these additional complex changes. On the other hand, the tight inverse relationship ($r = 0.94$) between receptor number and in vivo plasma insulin concentration in these two disparate protocols does argue for an important role of insulin in modulating cultured T-lymphocyte receptor display.

It is possible that alterations in insulin receptor display observed in these two experiments are a consequence of a shift of the lectin responsive population of T-cells, an increase in these cells by fasting, and a decrease by CSII. We made measurements to assure that the fraction of cells that were of T-cell pedigree present during the binding assay were similar for each patient during each study. Moreover, we could find no statistical differences in lectin-stimulated, radiolabeled thymidine incorporation irrespective of the in vivo plasma insulin level. This fact argues against the possibility that a simple shift in lectin-responsive cells explains observed changes in each patient. The high degree of correlation between the amount of change in plasma insulin concentration in two protocols and the amount and direction of the change in lectin-stimulated insulin receptor number argues strongly against the notion that an assay artifact or a change in other substances such as ketone bodies or fatty acids exerted a direct role in accounting for these data. We feel therefore that technical considerations do not explain the observed inverse relationship between plasma insulin concentrations and T-cell insulin receptor number.

An inverse relationship between insulin ligand and its receptor is an almost universal finding.[1,14–17] The crucial issue is the mechanism by which these regulatory signals are passed to the cell. It remains possible that these regulatory signals are passed to the lymphocyte by the binding event

itself. That would mean that there are a number of insulin receptors on unstimulated, circulating T-lymphocytes that are not detected by standard radioligand binding techniques[2,19,20] or insulin-supported T-cell biologic effects.[19,21] Changes in plasma concentration may lead to other alterations in the plasma space such as those related to levels of glucagon, fatty acids, ketone bodies, growth hormone, or catecholamines. Since T-lymphocytes have receptors in the circulation for some of these other hormones or neurotransmitters,[22,23] such changes may instruct the cell as to the number of insulin receptors it will ultimately display after in vitro cell activation. An attractive speculation derives from our laboratory's characterization of immunocompetent cell interaction for insulin receptor generation. Activated T-cells, when cultured with antigen naive B-cells devoid of insulin receptors, support B-cell synthesis of insulin receptors in the absence of other activating stimuli.[24] One can envision similar cell-to-cell cooperation between circulating monocytes, with their ubiquitous complement of insulin receptors, and the T-cell. In this formulation, plasma insulin levels are recorded by monocytes by virtue of ligand-receptor binding. This afferent signal is processed and could be passed along to the T-cell by means that do not require the presence of T-cell insulin receptors. By whatever mechanism, these data clearly demonstrate that T-lymphocyte insulin receptor regulation in vitro can be affected by the ambient plasma insulin level in vivo.

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