

Acute Regulation of Human Lymphocyte Insulin Receptors Analysis by the Glucose Clamp

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Abstract. The T lymphocyte insulin receptor model has been used to explore the regulation of insulin receptor appearance in that lymphocytes do not bear the insulin receptor in the circulation and thus are not amenable to regulation by virtue of ligand binding. Such cells synthesize insulin receptors when stimulated by antigen *in vivo* or *in vitro*. In these studies, the glucose clamp technique was employed to isolate perturbations in plasma glucose and plasma insulin as potential mediators of the regulation of the mitogen-induced T lymphocyte insulin receptor. Nondiabetic, normal weight individuals volunteered for 10 hyperglycemic clamp studies and nine euglycemic clamp studies with five individuals studied by both protocols. Hyperglycemia and hyperinsulinemia were created by the hyperglycemic clamp (basal plasma glucose was increased from 89 ± 2 mg/dl to 230 ± 2 mg/dl and an insulin of 99 ± 8 μ U/ml was reached). Blood was removed for isolation of T lymphocytes at 0, 1, 3, and 4 h of the clamped condition. After 1 h of hyperglycemia accompanied by an elevated plasma insulin, T cell insulin binding fell from 9.9 ± 0.9 pg/ 10^6 lymphocytes to 8.5 ± 0.9 pg/ 10^6 , and reached a nadir of $19 \pm 4\%$ at the conclusion of the clamp. Scatchard analysis of binding data from two of the subjects who underwent the hyperglycemic clamp demonstrated a reduction of the number of binding sites per cell without a change in the affinity of ligand for receptor. To separate the effects of glucose and insulin and the manner in which insulin is provided, the 4-h euglycemic clamp was performed in which fasting plasma glucose was maintained (95 ± 2 mg/dl) while constant hyperinsulinemia was created (80 ± 3 μ U/ml). Insulin binding

to activated, cultured T lymphocytes demonstrated a similar fall in insulin binding. Scatchard analysis of three additional studies again revealed a reduction in receptor number to $\sim 40\%$ of base line. These studies reveal that T cell insulin receptor regulation is achieved by hyperinsulinemia independent of the glucose level achieved. The reduction in insulin binding and receptor number could not be accounted for by variations in the strength of lectin stimulation, the time course of lectin response, or by the stress of the clamp itself. The effect of the clamp was specific for the lymphocyte insulin receptor in that the clamp had little effect on the interleukin II receptor activation marker. Acute changes in plasma insulin by the glucose clamp technique are perceived by the T lymphocyte and displayed in tissue culture by an alteration in lectin-induced insulin receptors. One can conclude that rapid changes in ambient *in vivo* insulin concentrations can regulate the synthesis of T lymphocyte insulin receptors generated *in vitro*.

Introduction

The human peripheral blood lymphocyte of thymic derived (T cell)¹ or bone marrow equivalent (B cell) pedigrees are devoid of cell membrane insulin receptors in the circulation unless presented with an appropriate antigenic stimulation (1-7). These cells may be induced to display these receptors in tissue culture upon presentation of antigen or mitogen. There is a tight temporal relationship between attainment and ultimate loss of the insulin receptor after cell activation (8). The presence of the insulin receptor on these cells plays an important role in hormonal modulation of lymphocyte immune function, enhancing cytotoxic T cell function, permitting mature cell differentiation, and maintaining the activated state of the lymphocyte after lectin or antigen challenge (3, 5, 8, 9). The receptor also permits ambient insulin to subserve the enhanced energy demands of activated T and B cells (10, 11). Characterization of control mechanisms that regulate the appearance

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1. Abbreviations used in this paper: anti-TAC, interleukin II receptor; B cell, bone marrow equivalent pedigrees; HBSS, Hanks' balanced salt solution; PHA-P, phytohemagglutinin; T cell, thymic-derived pedigrees.

of the insulin receptor on the lymphocyte is important for an understanding of hormonal modulation in general and the role of insulin in grading immune responses in particular.

Recently this laboratory has demonstrated that circulating lymphocytes obtained from patients with various disorders of carbohydrate metabolism may display intermediate numbers of insulin receptors after appropriate *in vitro* stimulation rather than the all-or-none relationships first felt to be operative (12). These static measurements did not permit assessment of whether the varied receptor number observed was an intrinsic property of the given disease or the result of changes in the milieu in which the lymphocyte was found and, thus, amenable to more precise regulation than could be permitted by cell activation alone. Alteration of the *in vivo* insulin concentration by a short-term fast or by insulin infusion was accompanied by regulation in lymphocyte insulin receptor numbers, which suggests that *in vivo* insulin concentrations might be the regulatory signal perceived by the lymphocyte and remembered in the tissue culture where cell activation and insulin receptor synthesis was effected (13). However, these previous studies were accompanied by many metabolic changes so that a more definitive conclusion concerning the regulatory role of plasma insulin levels could not be made. The purposes of the present studies were to determine whether the display of insulin receptors on lymphocytes could be acutely regulated by alterations of plasma insulin levels, and to discern by use of the glucose clamp technique whether fine regulation of the lymphocyte insulin receptor was the result of the changes in plasma glucose, plasma insulin, or both.

Methods

Patient population. Nondiabetic, normal weight, well informed, and consenting male volunteer subjects participated in this study. No subject had a history of diabetes mellitus, all were within 13% of their ideal body weight, all were euglycemic at the time of the study, and all had a normal fasting immunoreactive insulin. Mean age of all the subjects was 24 ± 1 yr. 10 hyperglycemic clamp studies and nine euglycemic studies were performed. Five subjects underwent both protocols to permit paired analysis. The clamp protocols in these five subjects were performed in random sequence with no two studies being performed within 1 mo of each other. Three additional control clamps were performed, one 4-h sham clamp, and two euglycemic clamps for analysis of the interleukin II receptor (anti-TAC) on lectin-activated T cells.

Glucose clamp protocols. The assumptions and mathematic algorithms that underlie the performance of both the hyperglycemic and euglycemic clamp have been well described (14, 15). In brief, subjects were studied in the Clinical Research Unit of this institution after an overnight fast. Plasma glucose concentrations were maintained near or at the "goal" glucose level by use of a negative feedback algorithm employing assays of "arterialized" blood glucose, measured every 2½ min for the first 30 min of the study and every 5 min for the remainder of the 240-min study (16). Blood for measurement of immunoreactive insulin was obtained at the same time points.

For the hyperglycemic clamp protocol, hyperglycemia at a level of 140 mg/dl above the fasting base-line concentration was created by a variable 20% glucose infusion adjusted by the negative feedback

algorithm after each plasma glucose measurement. For euglycemic clamp protocols, hyperinsulinemia was created by a primed/continuous insulin infusion while maintaining the plasma glucose level at base line and euglycemic levels by a variable glucose infusion.

Blood samples (20 ml) for measurement of T lymphocyte insulin receptor binding were obtained at 0-, 1-, 3-, and 4-h time points in both clamp protocols. In additional studies, 200 ml of heparinized blood was obtained at 0 and 4 h of the hyperglycemic clamp study in two subjects and in three subjects who were undergoing the euglycemic clamp for creation of full association binding isotherms to generate data for Scatchard analysis of the effect of the clamp on insulin receptor characteristics.

To determine the specificity of the insulin changes during the clamp on activated T cell insulin receptors, a 4-h sham clamp was performed. This study was conducted identically to the euglycemic clamps except that insulin was omitted from the preparation of the "insulin" infusion and 1 N NaCl was substituted for the "glucose" infusion. All catheters were placed in their usual veins and preplanned infusion rate changes were performed to recreate the atmosphere of a bona fide clamp.

Cell preparation. Standard and well-described techniques for recovering a highly T lymphocyte-enriched cell population from peripheral blood lymphocytes have been described and validated in previous manuscripts (5-7, 10, 11, 17, 18). In brief, heparinized blood obtained from clamped subjects was diluted 1:3 with Hanks' balanced salt solution (HBSS) buffered with 10 mM Hepes, and placed on a ficoll-hypaque density gradient. Passage of the mononuclear cells that were obtained from the gradient through nylon wool columns as previously described produced populations that were >95% T enriched (as determined by specific marker studies and by measurement of the OK-T3 surface marker by cytofluorography) and that were >98% viable by trypan blue exclusion. Importantly, the remaining cells that did not mark for T cell-specific surface antigens were not of the monocyte lineage as determined by esterase staining or use of a human monocyte-specific monoclonal fluorescent antibody. Lymphocytes that were obtained from patients who were undergoing one or the other clamp protocol were then placed into tissue culture under identical culture conditions to separate the impact of perturbations *in vivo* from any potential effects imposed by the culture conditions. Either clamp condition did not alter the yield of T cells, which was $\sim 0.67 \times 10^6$ /ml whole blood. 20×10^6 cells were placed in 30-ml Costar tissue culture flasks (Costar, Cambridge, MA) with RPMI 1640 culture media, enriched with 5% heat-inactivated, insulin-deplete, normal human serum, and buffered with 10 mM Hepes. To stimulate the synthesis of the T lymphocyte insulin receptor, cultures were pulsed with a final concentration of 5 µg/ml phytohemagglutinin-P (PHA-P) (Difco Laboratories, Detroit, MI) and cultured for 48 h in a 95% air/5% CO₂ gas mixture at 37°C in a humidified incubator. Cells were recovered from the cultures by centrifugation at 1,200 rpm in a Beckman desktop centrifuge (Beckman Instruments Inc., Fullerton, CA) for 10 min and washed once in enriched tissue culture media without lectin. Cells were placed at 10^7 /ml in HBSS-0.1% bovine serum albumin for the measurement of the insulin receptor.

To compare T lymphocyte binding data to the well-studied monocyte-macrophage model, these latter cells were recovered from parallel ficoll-hypaque gradients, adhered to glass Petri dishes warmed to 37°C, and washed twice with ice-cold HBSS. These cells were studied immediately after isolation without further lectin stimulation or cell cultures by construction of insulin association binding isotherms.

Insulin-binding assay. The basic assay technique, a modification of that described by Gammeltoft and Gliemann (18), has been described

previously and validated in several subsequent manuscripts (5, 10, 11, 17–19). The nature of nonspecific binding, the form of the Scatchard relationship, the success of separation of bound from free radiolabeled insulin, and the pharmacokinetic characteristics of the T cell insulin receptor have also been described previously (1–11). A single binding point was examined from cells obtained from eight hyper- and five euglycemic clamp studies using trace ^{125}I -iodoinsulin (1 nM). Enough cells were withdrawn at zero time and after 4 h of the clamp condition to construct complete association binding isotherms from two additional subjects undergoing the hyperglycemic and three subjects during the euglycemic clamp. In these latter studies, concentrations of ^{125}I -iodoinsulin from 0 to 60 ng/ml were presented to cells with and without excess (4 $\mu\text{g}/\text{ml}$) unlabeled single peak insulin. At least seven binding points were available for each accepted study. Insulin receptor characteristics were determined from analysis of the resultant Scatchard relationships (10, 12, 13).

Measurement of plasma glucose and immunoreactive insulin. The plasma glucose concentration during the clamp was measured using a glucose oxidase system on a Beckman glucose analyzer (Beckman Instruments Inc.). Insulin was measured by the Herbert modification (20) of the assay that was originally described by Yalow and Berson (21).

Measurement of T cell interleukin II receptors. In order to determine the specificity of the effects of the glucose clamp on the activated T cell insulin receptor, the effect of the clamp on a second T cell activation marker, the anti-TAC, was determined. Lymphocytes were obtained from two normal volunteers at the beginning and end of the euglycemic clamp protocol. Cell preparation and PHA cultures were performed identically to the lymphocyte insulin receptor studies. At the completion of the lectin cultures, the lymphocytes were harvested and placed at $2 \times 10^6/\text{ml}$. 100 μl of cells was mixed with 5 μl of a monoclonal antibody directed to the anti-TAC, prepared, and characterized by Uchiyama et al. (22), and was followed by incubation at 4°C for 30 min. 5 μl of fluorescein conjugated anti-mouse IgG (heavy and light chains) (Cappel Laboratories Inc., West Chester, PA) at 1:20 was added for an additional 30 min at 4°C. Fluorescence intensity and the number of stained cells were determined by analysis by the Ortho Systems 30L cytofluorograph (Ortho Diagnostic Systems Inc., Westwood, MA).

Thymidine uptake. It was important to assure that the conditions created by either glucose clamp study did not alter the degree of lectin stimulation or the lectin time course of action. T cells that were recovered from clamped subjects as described above at identical times as for receptor binding were placed at $10^6/\text{ml}$ in RPMI 1640 that was enriched by 1% normal human serum and buffered with 10 mM Hepes. 100 μl of the T cells was placed in each well of a flat-bottomed

microtiter tray (Flow Laboratories Inc., McLean, VA), to which was added 100 μl PHA-P to make a final concentration of 5 $\mu\text{g}/\text{ml}$. Culture proceeded for 48 h in a gas mixture of 95% air/5% CO_2 at 37°C, and was maintained by a humidified incubator. 4 h before harvest of the cells over glass fiber filters by a Mash II cell harvester (M.A. Bioproducts, Walkersville, VA), the cultures were pulsed with 25 μl (1 $\mu\text{Ci}/\text{well}$) of [^3H]thymidine. The glass fiber filters were counted by standard liquid scintillation spectrophotometry by a Beckman LS7000 (Beckman Instruments Inc.). For the time-course experiments, the 4-h thymidine pulse was analyzed daily for 5 d in consecutively harvested microtiter wells.

Results

Hyperglycemic clamp and insulin binding. The goal of these studies was to create hyperglycemia and hyperinsulinemia in normal subjects in vivo in order to examine the effects of these perturbations on in vitro insulin receptor binding to T lymphocytes. This goal was accomplished, as evidenced by the fact that the basal glucose concentration of 89 ± 2 mg/dl was raised to 230 ± 2 by the clamp technique, which was a change above base line of 140 ± 1 mg/dl, and that was nearly identical to the goal sought in each study. Hyperinsulinemia was also achieved, with peak immunoreactive insulin reached in the third to fourth hour of clamp, averaging 99 ± 8 $\mu\text{U}/\text{ml}$ (Table I and Fig. 1). Steady state hyperglycemia was achieved with an ever increasing level of hyperinsulinemia through the 4 h of clamp conditions. That steady state hyperglycemia was created by the clamp condition was demonstrated by the coefficient of variation of $4.0 \pm 0.3\%$ for the 10 studies, with no single study having a coefficient of variation $>5\%$.

Specific binding of ^{125}I -iodoinsulin to lectin activated, cultured T lymphocytes when freshly isolated from eight subjects undergoing the hyperglycemic clamp protocol is shown in Fig. 2. After 1 h of hyperglycemia accompanied by an elevated plasma insulin concentration, insulin binding fell from 9.9 ± 0.9 pg/ 10^6 lymphocytes to 8.5 ± 0.9 , a fall to 86% of control ($P < 0.02$; paired t analysis). Binding reached a nadir of $19 \pm 4\%$ of control or 1.9 pg of ^{125}I -iodoinsulin per 10^6 lymphocytes at the conclusion of the 4-h clamp. One can conclude from these data that hyperglycemia and/or hyperinsulinemia provide signals that lead to reduced insulin binding to T lymphocytes cultured ex vivo.

Table I. Performance of Normal Volunteers During 4-h Glucose Clamps

Clamp	Basal glucose	Clamped glucose	Δ Glucose	Basal IRI	Clamp IRI	Coefficient of variation in plasma glucose (0–240 min)
	mg/dl	mg/dl	mg/dl	$\mu\text{U}/\text{dl}$	$\mu\text{U}/\text{dl}$	%
Hyperglycemic (n = 10)	89 ± 2	230 ± 2	$+140 \pm 1$	9 ± 1	$99 \pm 8^*$	4.0 ± 0.3
Euglycemic (n = 9)	97 ± 2	95 ± 2	-1.0 ± 0.2	11 ± 1	$80 \pm 3^\ddagger$	4.4 ± 0.3

* Peak IRI achieved by the 4th h. \ddagger Mean IRI achieved over the 4-h study. IRI, immunoreactive insulin.

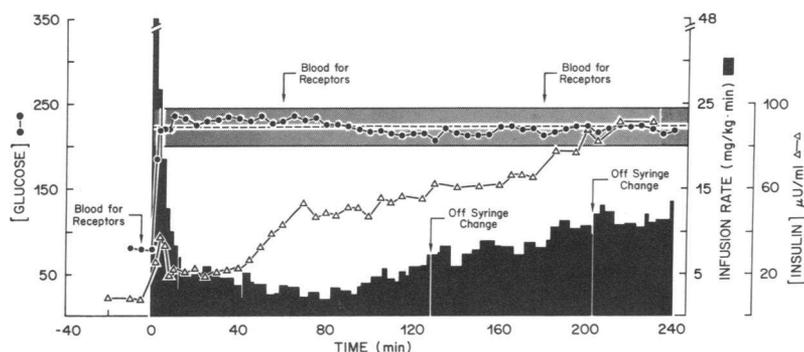


Figure 1. Graphic depiction of a representative 4-h hyperglycemic clamp. ●—●, plasma glucose concentration in mg/dl. Δ-Δ, plasma immunoreactive insulin in microunit per milliliter. The shaded bar graph represents the glucose infusion rate in milligram per kilogram per minute. The dotted line graphs the calculated hyperglycemic plasma glucose goal, while the grey shaded area shows the plasma glucose $\pm 10\%$ of the goal. The points at which blood was collected for measurements of T cell insulin receptors is marked. For the 4-h studies, several sets of syringes that contained the 20% glucose infusate had to be used, which necessitated pump syringe changes that are also marked.

In order to ascertain the locus of the reduced insulin receptor binding during hyperglycemic clamp conditions, lymphocytes from two subjects undergoing the hyperglycemic clamp protocol were studied over a wide range of radiolabeled ligand concentrations in order to construct an association binding isotherm. Scatchard analysis (Fig. 3) from one of these subjects demonstrates that diminished binding is a consequence of a reduced number of receptor sites on the cell. In this subject, plasma glucose was increased from 80 mg/dl to a mean of 221 ± 12 mg/dl during the 4-h clamp. Immunoreactive insulin was increased from $14 \mu\text{U/ml}$ at the zero time, at which T lymphocytes were first sampled, to $129 \mu\text{U/ml}$ when lymphocytes were next sampled for analysis of insulin receptor binding. In this study, the perturbation that was achieved during the 4-h hyperglycemic clamp did not affect the slope of binding, and thus did not affect the affinity of the ligand for its receptor (2.8 nM to cells removed at zero time as compared with 2.1 nM after 4 h of clamp). The intercept, directly related to the number of sites, was clearly reduced by in vivo perturbations in glucose and in insulin achieved during the hyperglycemic clamp. The number of insulin receptor binding sites that were synthesized in tissue culture in response to lectin stimulation was reduced when in vivo conditions were perturbed by the hyperglycemic clamp in this subject from 6,023 sites per cell to 2,333 sites per cell. The impact of hyperglycemia and progressive hyperinsulinemia achieved during the clamp on ex vivo, tissue culture-activated T lymphocyte insulin receptor binding site number was similar in the second patient studied as that detailed here. One can conclude from

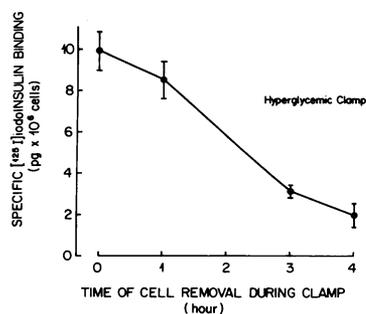


Figure 2. Specific binding of trace ^{125}I -iodoinsulin to PHA stimulated T lymphocytes removed during the 4-h hyperglycemic clamp ($n = 8$).

the hyperglycemic clamp protocol that creation of hyperglycemia and/or hyperinsulinemia is perceived by T lymphocytes in vivo and displayed in tissue culture such that there is a sharp reduction in lectin activated insulin receptor sites.

Euglycemic clamp and T lymphocyte insulin binding. It could be argued that the progressive fall in insulin receptor binding sites on T lymphocytes that was achieved during the hyperglycemic clamp and observed in tissue culture 48 h after removal of the cell from the patient was entirely a consequence of the steady rise of immunoreactive insulin during the hyperglycemic clamp. To explore this potential explanation of the data and to examine the potential regulatory role of the hyperglycemia achieved during the hyperglycemic clamp, the euglycemic clamp was employed. The goal of the euglycemic clamp, then, was to achieve steady state elevations in immunoreactive insulin to test the role of rate of rise of insulin vs. the magnitude of the insulin concentration in plasma space while maintaining basal glucose, thus removing this variable as a potential receptor regulator. These goals were met as demonstrated in Table I and Fig. 4. The mean clamp glucose concentration from 0 to 240 min was 95 ± 2 mg/dl, which was within 1.0 ± 0.2 mg/dl of basal glucose by paired analysis of each study. While achieving this steady state, clamp glucose concentration equivalent to fasting levels during 4 h, a steady

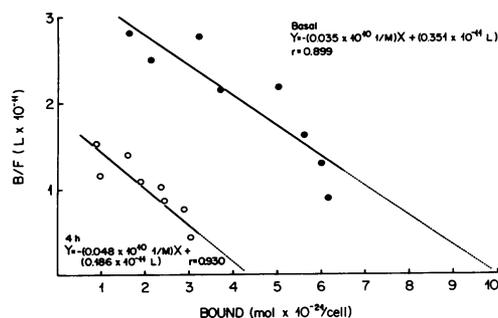


Figure 3. Scatchard transformation of a full association binding isotherm from a subject undergoing a 4-h hyperglycemic clamp. ●, Data derived from T lymphocyte specific insulin binding on cells removed at zero time. ○, Data derived from cells removed at the 4th h of clamped conditions.

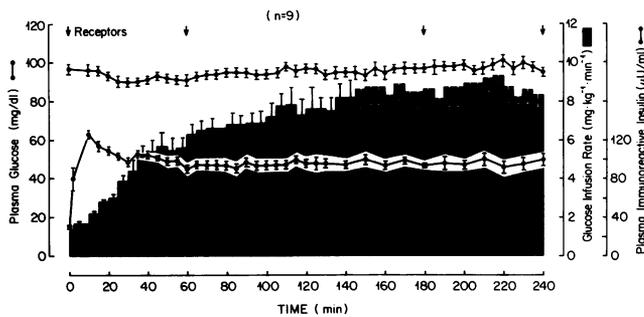


Figure 4. Graphic depiction of the variables measured during nine euglycemic clamp studies. Plasma glucose in milligram per deciliter is graphed above the bar graph, while plasma immunoreactive insulin in microunit per milliliter is graphed within the bar graph. The bar graph shows the glucose infusion rate. The time points at which cells were removed for analysis of the T lymphocyte insulin receptor are marked.

state elevation in immunoreactive insulin to $80 \pm 3 \mu\text{U/ml}$ was achieved. Variations in plasma glucose concentration during the clamp were small, as a coefficient of variation during the 4-h study of $4.4 \pm 0.2\%$ was achieved.

Specific iodinsulin binding to cultured, activated T lymphocytes declined on cells removed over 4 h in the euglycemic clamp protocol (Fig. 5; the data derived from the hyperglycemic clamp protocol are included in this figure for comparison). These new data represent single, trace iodinsulin binding studies to five subjects, all of whom had participated in the hyperglycemic clamp protocol as well, allowing comparison of basal, zero time binding in these groups of subjects. Basal binding of $9.7 \text{ pg}/10^6$ lymphocytes before the hyperglycemic clamp was not statistically significant from the $9.1 \text{ pg}/10^6$ found in the same patients before the euglycemic clamp. There was a steady fall in specific receptor binding to freshly isolated, then lectin activated T lymphocytes in a similar pattern after euglycemic clamping as that observed in the hyperglycemic clamp protocol. Again, significant reductions in insulin receptor binding in vitro ($66 \pm 7\%$; $P < 0.01$ of control) were observed after only 1 h of in vivo creation of hyperinsulinemia in this clamp protocol. The nadir achieved at 4 h of $27 \pm 6\%$ was not significantly different from that achieved after hyperglycemic clamping. Thus, T cell receptor regulation was achieved by a

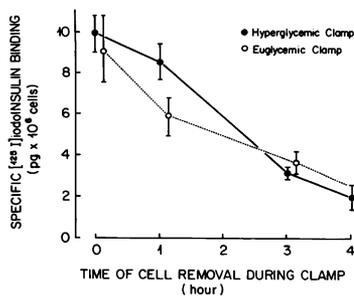


Figure 5. Specific binding of trace ^{125}I -iodoinsulin to PHA stimulated T lymphocytes removed during the 4-h euglycemic clamp ($n = 5$) \circ . The results from the 4-h hyperglycemic clamp are depicted as \bullet for comparison.

given level of hyperinsulinemia rather than the rate of rise of insulin. One can also conclude that hyperglycemia was unnecessary for the diminution in T cell receptor binding observed after acute perturbations in plasma insulin concentration.

The impact of the acute creation of in vivo hyperinsulinemia during glucose clamping on in vitro generation of lectin-stimulated, T cell insulin receptor characteristics was further evaluated by Scatchard analysis of full association binding isotherms that were derived from binding data from the three additional subjects undergoing the euglycemic clamp. As in the hyperglycemic study, it can be shown that the effect of hyperinsulinemia is at the level of the number of insulin receptor binding sites measurable on the lymphocyte membrane. T lymphocyte insulin receptor sites fell from $6,054 \pm 1,523$ measured before clamping began to $3,955 \pm 1,083$ after 4 h of hyperinsulinemia in these three subjects. This effect of hyperinsulinemia that was achieved during the clamp on cultured lymphocyte insulin receptor number was observed in each of the three studies, averaging $36 \pm 6\%$ fewer sites (paired analysis) after 4 h of clamp.

Insulin receptor binding to monocyte-macrophages. To compare the results obtained in this study with previously published information with respect to the monocyte-macrophage, insulin receptor binding was studied on these latter cells on the day of glucose clamping. In data not shown, the shape of the monocyte binding curve in our hands was curvilinear, and similar to that reported by a multiplicity of laboratories. Binding fell to $\sim 11\%$ of control during the 4 h of either clamped study. In the hyperglycemic clamp protocol, for example, monocyte binding fell from $11.9 \pm 0.9 \text{ pg}/10^6$ monocytes at zero time to $8.8 \pm 0.7 \text{ pg}/10^6$ monocytes at 1 h ($P < 0.01$), and fell to a nadir of 1.26 ± 0.2 at hour four ($P < 0.001$).

Specificity controls. Activated T cell insulin receptor binding to cells that were removed at 0, 2, and 4 h during a sham

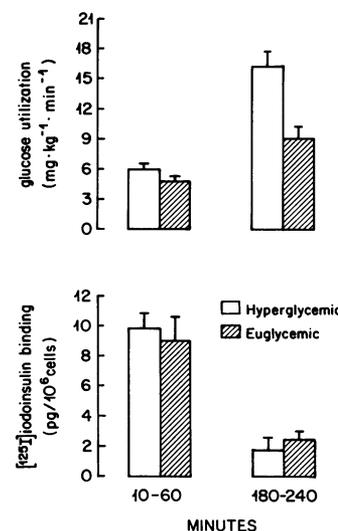


Figure 6. Comparison of the amount of specific insulin binding to T lymphocytes in the first hour of each clamp protocol with whole body insulin effect as an estimate of the glucose utilization rates calculated during the first and fourth hour of each clamp.

euglycemic clamp was similar and not reduced (3.6 pg/10⁶ cells, zero time; 3.9 pg/10⁶, 2 h; 3.5 pg/10⁶, 4 h). The number of anti-TAC staining T cells was slightly reduced (43% at zero time to 37% at the completion of the clamp) in one study and was unchanged in the second.

Lectin stimulation and time course. T cells studied at 0, 2 and 4 h of either clamp and cultured with PHA for 48 h exhibited similar thymidine uptake (99,782±3,812 cpm). There were no statistical differences between the mean uptake of any time point or any time from this mean of all the data. Conditions created by either clamp did not alter the time to peak PHA response for these human T lymphocytes which occurred at 48–60 h.

Discussion

Study of the insulin receptor on various circulating cells has advanced an understanding of peptide receptor chemistry in general and insulin receptor biology in particular. The circulating T lymphocyte has several useful features that make it a relevant model with which to explore the phenomenon of membrane-bound receptor regulation and potential mechanisms thereof. In some ways, the T lymphocyte insulin receptor is quite similar to that found on a wide range of body tissues, including the classical insulin target tissues such as liver, muscle, and fat, in that the insulin binding site on the surface of the T lymphocyte demonstrates high specificity, saturability, and high affinity (5). Binding of the insulin ligand to its receptor carries important immunobiologic consequence and also enhances classically described pathways of intermediary metabolism (3–5, 9–11). In contrast to cultured cell lines, such as the IM-9 lymphoblast, preliminary evidence demonstrates that processing of the ligand receptor complex by the T cell is similar to that described for adipocytes with internalization, lysosomal processing, and reinsertion into the membrane from the cytosol (23, 24).

On the other hand, the T lymphocyte model has several unique features which make it particularly relevant for study of insulin receptor regulation. The T cell does not have an insulin receptor in peripheral blood in usual circumstance (1–7). The insulin receptor is only measurable on the cell when the subject is exposed to substantial antigenic challenge, such as is achieved by transplantation in humans or animals, or when the cell is removed from the circulation and stimulated in tissue culture with antigen or mitogen. In these latter circumstances, T lymphocyte insulin receptor synthesis is initiated by the stimulus and is measurable on the cell surface in ~24 h. Thus, in a simple way, alteration of the ligand in the circulation cannot directly modulate the appearance of insulin receptor on the T cell by binding in vivo, for this cell has no measurable receptor in the circulation to permit such binding. Moreover, the insulin receptor on the T lymphocyte is generated on the cell in tissue culture under highly controlled conditions. Using the T cell model, we recently demonstrated a diminished insulin receptor number on T lymphocytes that

were obtained from obese, nondiabetic subjects and from subjects with type II noninsulin-requiring diabetes mellitus (12). These studies were conducted as static measurements of fasting samples in which no perturbation of the plasma milieu was desired or created. Two interpretations of the available data obtained in these static, unperturbed conditions were advanced. Either the reduced receptor number reflected a genetically programmed defect or T cells have a capacity to detect changes in the ambient plasma despite the fact that no insulin receptors were present.

In a second series of studies, the concept that the T lymphocyte could discern and respond to modulation of the ambient plasma conditions was explored (16). Plasma insulin concentrations were modulated in a chronic fashion with an assessment of subsequent development of insulin receptors on T lymphocytes that were isolated during the perturbation and then stimulated in tissue culture. An 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 was observed. The T lymphocyte, indeed, has the capacity directly or indirectly to perceive chronic changes in the ambient insulin concentration and to “down regulate” or to alter its receptor generation capacity.

The protocols used in the previous analysis of the regulation of in vitro generation of the T lymphocyte insulin receptor by in vivo modulation of insulin were accompanied by a range of other changes that might indeed have affected the nature of receptor regulation on this unique cell. Although it was argued that the tight inverse relationship between receptor number and in vivo plasma insulin concentration established the important role of insulin as the modulating agent, changes in plasma glucose among other hormonal and metabolic changes could also have contributed to the regulation. The glucose clamp techniques utilized in the present studies were designed to more strictly isolate glucose and insulin as potential modulating forces for the in vitro generation of the T lymphocyte insulin receptor. The studies reported here demonstrate that creation of steady state hyperglycemia at ~140 mg/dl above fasting basal levels, accompanied by elevated levels of immunoreactive insulin, leads to a progressive reduction in the number of insulin receptors displayed on cultured, lectin-activated T lymphocytes. That virtually identical, progressive reductions in the number of T cell insulin receptor sites occurred after the euglycemic clamp in which glucose was maintained at the basal level while steady state hyperinsulinemia was created, argues strongly for the independence and primacy of hyperinsulinemia as the modulating factor in T lymphocyte receptor display. Further, one can see that the regulating signal relates more closely to the absolute concentration of insulin in plasma space rather than to the rate of rise of the hormone.

The short term adaptive response of the insulin receptor in general and that following the acute regulation created by the glucose clamp technique in particular have been studied previously on erythrocytes (25) and monocytes (26). Insel et al. (26) found that insulin binding diminished by 36% after 5

h of hyperinsulinemia and hyperglycemia. Careful Scatchard analysis and average affinity analysis of the erythrocyte binding data implicated a fall in receptor affinity to explain the reduced binding. In contrast to the Insel data (25) on the red cell are those of Soman and DeFronzo (26) on the monocyte. Using one of several euglycemic clamp protocols, Soman and DeFronzo (26) found monocyte binding also to be diminished after 5 h of clamping, but the diminution in binding was entirely a consequence of the reduction of receptor numbers with no change in receptor affinity. Both of these cell types vary from the T lymphocyte model in that both erythrocytes and monocytes bear the insulin receptor in the circulation and thus are amenable to direct regulation by the binding of the ligand to the cell membrane in vivo, and the binding of insulin to neither cell carries physiologic consequence. Exploring the T lymphocyte model, we have found that the insulin-induced regulation of the in vitro generation of insulin receptors is directed at controlling the number of receptors on the T cell membrane, which may alter the impact of insulin in modulating the immunobiologic role of the lymphocyte, which itself does not participate in control of the level of insulin concentration.

Two controls were performed to determine whether the effect of the glucose clamp on regulation of the activated T cell insulin receptor was specific for the hormonal changes that were induced by the clamp on the one hand or for the T cell insulin receptor on the other. That there were no changes in activated T cell insulin receptor number when lymphocytes were obtained during a sham clamp that was performed in every way identical to a 4-h euglycemic but without plasma glucose or plasma insulin changes, argues strongly that the stress of the clamp itself cannot explain the T cell insulin receptor changes. More important is the possibility that acute changes in plasma insulin reduce the capacity of the activated lymphocyte to synthesize a number of different receptor molecules, which are all known to be activation markers. In the second series of control experiments, acute alteration of plasma insulin had little or no effect on the appearance of the anti-TAC on lectin-activated T cells. One can conclude that changes in plasma insulin specifically regulate the appearance of the activated T cell insulin receptor.

Three distinct mechanisms can now be described for the regulation of T lymphocyte insulin receptors. Firstly, antigen provides an all-or-none signal for de novo synthesis of insulin receptors, the first event of which is translation of nuclear messenger RNA (17). In controlled in vitro circumstances, provision of ligand to pure cultures of receptor-negative T cells undergoing antigen stimulation fails to further regulate receptor display (4). A second mechanism reflects the "down regulation" observed when ligand is presented to T cells bearing their full complement of insulin receptors (4). Our studies now provide strong support for a third mechanism of receptor regulation that is related to alterations of ambient insulin in vivo that are perceived by circulating, receptor-negative T cells and remembered in tissue culture by a means which perforce cannot involve ligand binding. Based on the observation that T cells and B cells can interact to regulate B cell insulin

receptor number, one may construct a model hypothesis to potentially explain how a receptorless T cell receives signals concerning ambient insulin concentrations. In this construct the receptor-bearing monocyte discerns insulin concentrations in vivo and instructs the T cell in a manner similar to antigen processing and presentation. This hypothesis, if proven, provides further links between hormone and immune control mechanisms.

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References

1. Krug, U., F. Krug, and P. Cuatrecasas. 1972. Emergence of insulin receptors on human lymphocytes during in vitro transformation. *Proc. Natl. Acad. Sci. USA.* 69:2604-2608.
2. Schwartz, R. H., A. R. Bianco, B. S. Handwerker, and C. R. Kahn. 1975. Demonstration that monocytes rather than lymphocytes are the insulin-binding cells in preparations of human peripheral blood mononuclear leukocytes. Implications for studies of insulin-resistant states in man. *Proc. Natl. Acad. Sci. USA.* 72:474-478.
3. Kumagai, J.-I., H. Akiyama, S. Iwashita, H. Iida, and I. Yahara. 1981. In vitro regeneration of resting lymphocytes from stimulated lymphocytes and its inhibition by insulin. *J. Immunol.* 126:1249-1254.
4. Ercolani, L., T. J. Brown, and B. H. Ginsberg. 1984. Tunicamycin blocks the emergence and maintenance of insulin receptors on mitogen activated human T-lymphocytes. *Metab. Clin. Exp.* 33:309-317.
5. Helderman, J. H., and T. B. Strom. 1977. The emergence of insulin receptors upon alloimmune cells in the rat. *J. Clin. Invest.* 59:338-344.
6. Helderman, J. H., and T. B. Strom. 1978. The specific insulin binding site on T and B lymphocytes: a marker of cell activation. *Nature (Lond.)* 274:62-63.
7. Helderman, J. H., T. C. Reynolds, and T. B. Strom. 1978. The insulin receptor as a universal marker of activated lymphocytes. *Eur. J. Immunol.* 8:589-595.
8. Helderman, J. H., T. B. Strom, and A. Dupuy-d'Angeac. 1979. A close relationship between cytotoxic T lymphocytes generated in the mixed lymphocyte culture and insulin receptor bearing lymphocytes: enrichment by density gradient centrifugation. *Cell. Immunol.* 46:247-258.
9. Strom, T. B., and J. D. Bangs. 1982. Human serum-free mixed lymphocyte response: the stereospecific effect of insulin and its potentiation by transferrin. *J. Immunol.* 128:1555-1559.
10. Helderman, J. H. 1981. The role of insulin in the intermediary metabolism of the activated thymic derived lymphocyte. *J. Clin. Invest.* 67:1636-1642.
11. Helderman, J. H. 1983. T cell cooperation for the genesis of B cell insulin receptors. *J. Immunol.* 131:644-650.
12. Helderman, J. H., and P. Raskin. 1980. The T-lymphocyte

insulin receptor in diabetes and obesity: an intrinsic binding defect. *Diabetes*. 29:551-557.

13. Helderman, J. H., A. Pietri, and P. Raskin. 1983. In vitro control of T lymphocyte insulin receptors by in vivo modulation of insulin. *Diabetes*. 32:712-717.

14. DeFronzo, R. A., J. D. Tobin, and R. Andres. 1979. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am. J. Physiol.* 237:E214-E223.

15. McGuire, E. A. H., J. H. Helderman, J. D. Tobin, R. Andres, and M. Berman. 1976. Effects of arterial versus venous sampling on the analysis of glucose kinetics in man. *J. Appl. Physiol.* 41:565-73.

16. Helderman, J. H., D. Elahi, D. K. Andersen, G. S. Raizes, D. Shocken, and R. Andres. 1983. Prevention of the glucose intolerance of thiazide diuretics by maintenance of body potassium. *Diabetes*. 32:106-111.

17. Helderman, J. H., and T. B. Strom. 1979. Role of protein and RNA synthesis in the development of insulin binding sites on activated thymus-derived lymphocytes. *J. Biol. Chem.* 254:7203-7207.

18. Gammeltoft, S., and J. Gliemann. 1973. Binding and degradation of ¹²⁵I-labelled insulin by isolated rat fat cells. *Biochim. Biophys. Acta.* 320:16-32.

19. Gliemann, J., K. Østerlind, J. Vinten, and S. Gammeltoft.

1972. A procedure for measurement of distribution spaces in isolated fat cells. *Biochim. Biophys. Acta.* 286:1-9.

20. Herbert, V., K. S. Lau, C. W. Gottlieb, and S. J. Bleicher. 1965. Coated charcoal immunoassay of insulin. *J. Clin. Endocrinol. Metab.* 25:1375-85.

21. Yalow, R. S., and S. A. Berson. 1960. Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.* 39:1157-75.

22. Uchiyama, T., S. Broder, and T. A. Waldmann. 1981. A monoclonal antibody (Anti-TAC) reactive with activated and functionally mature human T-cells. 1. Production of anti-TAC monoclonal antibody and distribution of TAC (+) cells. *J. Immunol.* 126:1393-1397.

23. Marshall, S., and J. M. Olefsky. 1979. Effects of lysosomotropic agents on insulin interactions with adipocytes. Evidence for a lysosomal pathway for insulin processing and degradation. *J. Biol. Chem.* 254:10153-10160.

24. Helderman, J. H. 1984. Cellular processing of the T-lymphocyte insulin receptor. *Clin. Res.* 32:398A. (Abstr.)

25. Insel, J. R., O. G. Kolterman, M. Saekow, and J. M. Olefsky. 1980. Short-term regulation of insulin receptor affinity in man. *Diabetes*. 29:132-139.

26. Soman, V. J., and R. A. DeFronzo. 1980. Direct evidence for downregulation of insulin receptors by physiologic hyperinsulinemia in man. *Diabetes*. 29:159-163.