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PHYTOHEMAGGLUTININ (PHA) ACTIVATED HUMAN T-LYMPHOCYTES: CONCOMITANT APPEARANCE OF INSULIN BINDING, DEGRADATION AND INSULIN-MEDIATED ACTIVATION OF PYRUVATE DEHYDROGENASE (PDH)

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SUMMARY: Binding and degradation of $A_{14}{}^{125}I$-Insulin as well as the effect of insulin on pyruvate dehydrogenase (PDH) activation were studied in non-stimulated and phytohemagglutinin (PHA)-stimulated thymic-derived lymphocytes (T-lymphocytes) of man under varying conditions of time, temperature, and cell concentration. The nonstimulated viable T-lymphocytes exhibited neither binding, degradation, nor PDH activation in response to insulin. With PHA stimulation, a time and temperature-dependent binding was noted in T-lymphocytes which paralleled the appearance of cell-associated insulin degrading activity. Concomitant with the emergence of insulin binding and degrading activities in these cells, PDH activation was observed which was responsive to as little as 5.0 μU/ml of insulin. We conclude that in PHA-activated T-lymphocytes of man the process of insulin binding and degradation is closely related to insulin sensitive activation of PDH. These activated cells may serve as a useful model in which to study insulin binding and processing, as well as effects of insulin on postreceptor events.

INTRODUCTION: Various human tissues have been used to examine insulin binding and metabolism. However, these tissues are either not easily obtainable from patients (e.g., adipocytes), require large volumes of blood (e.g., monocytes), or their physiologic significance in insulin metabolism is not clear (e.g., red blood cells). Recently, evidence has been presented to suggest that human peripheral T-lymphocytes when activated with mitogens may provide a readily available tissue source for examining insulin binding and its actions (1-4).

Human T-lymphocytes normally comprise 80-85% of the circulating peripheral mononuclear cells. Normal circulating T-lymphocytes are incapable of either binding or responding to insulin (2-7). However, when stimulated in short-term culture by mitogens, T-lymphocytes develop insulin receptors (2-7). Along with the emergence of the insulin receptor, the cells become responsive

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to insulin. Insulin has been found to enhance both glucose uptake and oxidation in insulin receptor positive but not in insulin receptor negative T-lymphocytes (4,7). Mitogen-stimulated T-lymphocytes therefore may serve as a valuable tool for examining insulin action at both the receptor and postreceptor levels.

Since insulin binding and processing, including insulin degradation, may be related to insulin action (8-10) we investigated the role of insulin receptor generation and insulin degradation with the effect of insulin on mitochondrial pyruvate dehydrogenase (PDH) of peripheral T-lymphocytes. Our results show a hitherto unknown relationship between insulin binding, its degradation and PDH response to insulin in phytohemagglutinin (PHA)-stimulated T-lymphocytes of man.

MATERIALS AND METHODS

Materials

Fetal bovine serum, L-glutamine, and RPMI 1640 were purchased from Gibco (Grand Island, New York). Highly purified porcine insulin was kindly provided by Dr. Ronald Chance of Eli Lilly (Indianapolis, Indiana). Phytohemagglutinin (PHA) was purchased from Difco Laboratories (Detroit, Michigan); and radiolabeled compounds were from Amersham (Arlington, Illinois). All other chemicals were of reagent grade and obtained from commercial sources as previously described (11).

Iodination of $A_{14}{}^{125}I$-Insulin

Insulin was iodinated by the chloramine T method and purified to carrier-free monoiodinated $A_{14}{}^{125}I$-insulin with a specific activity of 360 μCi/μg by purification on a high performance liquid chromatograph (HPLC) as described previously (12).

Separation of T-lymphocytes from Peripheral Blood

T-lymphocytes were separated from peripheral blood by the sheep red blood cell (RBC) rosetting techniques described by Herrod and Buckley (13). Aliquots of blood diluted 1:2.5 with saline were underlayered with 0.9% Ficoll hypaque prior to centrifugation at 400xg. The mononuclear cells were aspirated and washed three times in phosphate buffered saline (PBS) containing 2% newborn calf serum (NCS). Aliquots of cells ($2\text{-}4 \times 10^6$) were then incubated for one hour at 4°C with 1% 2-aminoethylisothiouronium bromide hydrobromide-treated sheep RBCs. The mixture was then underlayered with Ficoll hypaque, and centrifuged at 400xg for 30 minutes. The supernatants were discarded and pellets containing rosettes were incubated with lysing buffer (Tris-NH_4Cl) for 10-15 minutes at 37°C. Cells were then washed 3 additional times with PBS-2% NCS and resuspended in culture media (RPMI 1640 containing 10% newborn calf serum, 1% glutamine).

T-lymphocyte Cultures

Cells were cultured in 25 ml flasks at 1×10^6 cells/ml (total 10×10^6/flask) in culture media and in the presence or absence of 1 μg/ml of purified PHA at 37°C under 5% CO_2. On the day of the experiment, cells were centrifuged at 350xg for 10 minutes, 4°C, and resupended in Hanks buffer (Gibco) containing 1% bovine serum albumin (BSA). Cell number was determined and viability examined. Cells with viability less than 95% were discarded.

T-lymphocyte Binding

Following culture, cells were washed 3x with PBS-2% NCS and resuspended in Hanks-1% BSA buffer, counted, and checked for cell viability. Aliquots of cells ($5x10^6$) were added to tubes containing at final volume 0.75ml Hanks-1% BSA; 187,500 cpm ^{125}I-Insulin; and 0 or 10^5 ng/ml unlabeled insulin for determination of total and nonspecific binding, respectively. Cells were then incubated at the temperatures and times described in the figure legends. At the end of the incubation period, triplicate aliquots (0.2ml) were removed and layered over 0.7ml 1:2 dinonyl-dibutyl phthalate, and centrifuged at 10,000xg for 2 minutes to separate free from bound hormone. The supernatants were aspirated; the portion of tubes containing the pellets cut; and cell-associated radioactivity determined. Specific binding was calculated as total ^{125}I-Insulin binding minus nonspecific binding (10^5ng/ml insulin).

Insulin Degradation

Degradation of insulin by T-lymphocytes was determined by preincubating cells with ^{125}I-Insulin for 2 hours at 15°C. At the end of the binding period, cells were centrifuged at 350xg for 10 minutes at 4°C, and the cellular pellet washed twice in 1% BSA-Hanks buffer. Cells were then resuspended in 1% BSA-Hanks buffer and incubated at 37°C for varying time periods. At the end of the incubation, triplicate aliquots of 0.2ml cellular mixture were added to tubes containing 0.8 ml of 0.3% BSA and 1.0 ml of 10% TCA. Cell-associated degradation was calculated from the ratio of radioactivity in the solubilized and precipitated fractions.(14)

PDC Activity

Following 72 hours of culture, mitogen-stimulated cells and their nonstimulated controls were centrifuged and washed as described above for insulin binding. Aliquots of $25x10^6$ cells were added to incubation tubes containing 0.2% BSA-Hanks buffer and in the presence or absence of insulin. The cells were then incubated for 30 minutes at 37°C and centrifuged for 10 minutes at 350xg, 4°C. Supernatants were discarded and the pellets quick-frozen in a dry ice-acetone bath.

'Initial' (PDH_a) and 'total' (PDH_t) activities were determined from the extracts of the frozen cellular pellets according to a modification of the methods of Olson et al. (15). Each pellet ($25x10^6$ cells) was resuspended in 100 mM phoshate-tris buffer, pH 7.4, containing 2mM dithiothreitol. Aliquots (0.4ml) were removed in duplicate for measurement of the PDH_a and PDH_t. The samples used for the determination of the PDH_a were added to tubes containing 25 mM NaF, 1.0 mM dichloroacetate, 1.0 mM $CaCl_2$, 10 mM $MgCl_2$, 2mM EDTA, 100mM phosphate buffer, 100 µl rabbit serum, 20 µg/ml aprotinin, and 10 µg/ml phenylmethyl sulfonyl fluoride (PMSF). These conditions provide for inhibition of the PDH kinase-phosphatase and prevent proteolysis. The samples were then immediately frozen in a dry-ice acetone bath. Other aliquots (0.4ml), removed from the original homogenate for determination of PDH_t, were added to a second set of tubes which contained the proteolytic inhibitors (aprotinin, PMSF, rabbit serum), the kinase inhibitor (dichloroacetate), and activators of the PDH phosphatase (Ca^{2+}, Mg^{2+}). Kinase inhibition and phosphatase activation provide for maximal activation of the PDH. Following a 30 minute incubation at 37°C, NaF and EDTA were added, and the samples vortexed and quick-frozen.

For measurement of PDH activity, 0.5 ml aliquots of the PDH_a and PDH_t were added to Erlenmeyer flasks containing 100mM phosphate, 10mM NAD^+, 1mM CoA, 1mM thiamine pyrophosphate, 10mM $MgCl_2$, 5mM pyruvate, $9x10^5$ CPM [1-^{14}C]-pyruvate, and 0.025% triton-X 100. The flasks were then sealed with serum stoppers containing center wells and the reaction continued for 8 minutes. The reaction was terminated with addition of 0.5 ml 1N HCl and the $^{14}CO_2$ liberated was trapped in 0.3 ml of phenethylamine contained in the center wells. The flasks were then gently agitated at room temperature for one hour and the center wells transferred to 10 ml scintillation fluid (Liquiscint) for counting. Backgrounds were determined from $^{14}CO_2$ produced at 0 time of the assay. PDH_a and PDH_t were expressed as nmol $^{14}CO_2$

produced/min/# of cells. The activation state (the proportion of the enzyme in its active form) was calculated from the ratio of PDH_a/PDH_t.

RESULTS

The development of insulin binding and the appearance of insulin degrading activities have been examined in PHA-stimulated and nonstimulated T-lymphocytes and the results are presented in Figure 1. As can be seen, ^{125}I-Insulin binding in PHA-stimulated cells was measurable within 24 hours of culture and reached a plateau after 72 hours. However, cells cultured for identical time periods but in the absence of PHA were incapable of binding or degrading insulin. Our results thus suggest a possible relationship between the emergence of the insulin receptor in activated T-lymphocytes and the ability of these cells to degrade insulin. This relationship is further demonstrated by the data illustrated in Figure 2. As can be seen in this figure, both insulin binding and degradation respond to concentration of cells in a linear fashion.

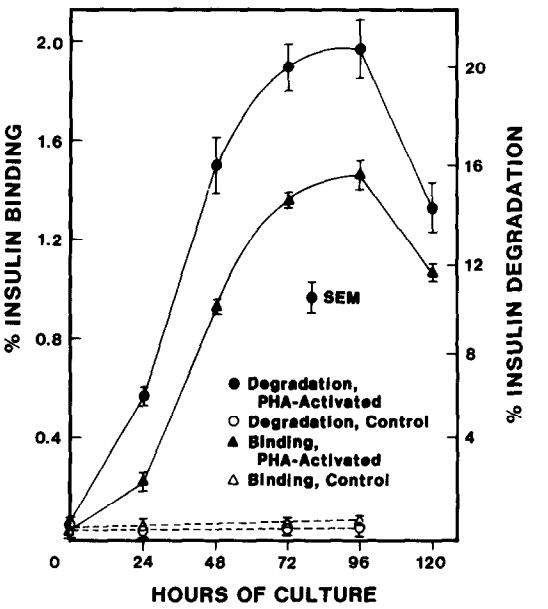
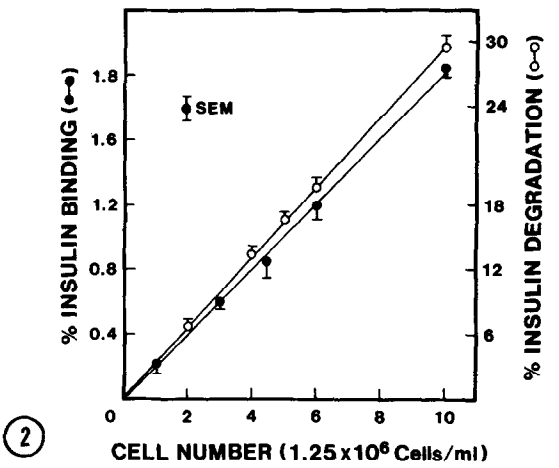


Figure 1: Development of Insulin Binding and Degrading Activity. T-lymphocytes were cultured in the presence or absence of 1 µg/ml PHA for the time periods designated. Upon removal from culture, cells were washed and incubated for 2 hours at 15°C for measurement of % specific insulin binding as described in the "Methods." Cell-associated degradation was determined following an additional 90 minute, 37°C incubation (see "Methods"). Specific binding is reported as % binding per 1.25×10^7 cells and degradation as % degradation per 5×10^6 cells/ml. Values are the mean ± SEM for 3 determinations.



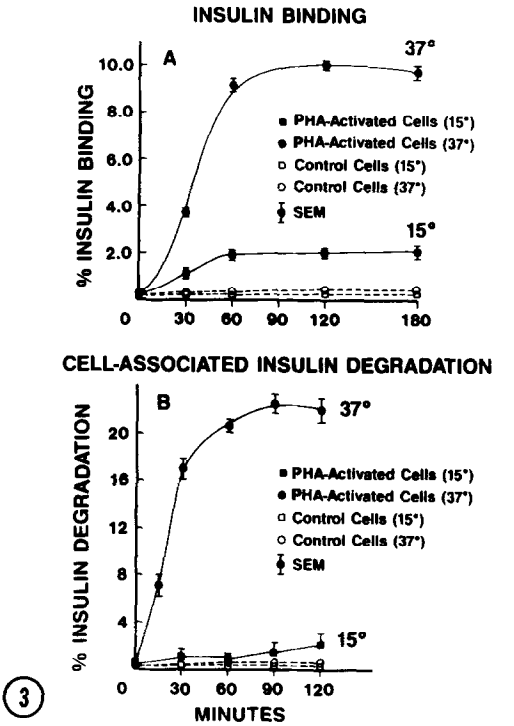


Figure 2: Effects of Cell Concentration on Insulin Binding and Degradation. Specific ^{125}I-Insulin binding and degradation were determined as described in the "Methods" and the legend of Figure 1. Cells consisted of PHA-stimulated T-lymphocytes cultured for 72 hours. The values represent the mean ± SEM for 6 determinations from 2 separate experiments.

Figure 3: Effects of Time and Temperature on Insulin Binding and Degradation. Panel A shows % specific binding of ^{125}I-Insulin in PHA-stimulated and nonstimulated T-lymphocytes at 15°C and 37°C for varying lengths of time. Panel B shows the effects of time and temperature on insulin degrading activity following an initial 120 minute, 15°C incubation of cells with ^{125}I-Insulin. The results are the mean ± SEM of 3 determinations and are representative of values observed on 3 separate occasions.

Figure 3, Panels A and B show the effects of time and temperature on insulin binding and cell-associated insulin degradation, respectively. As is demonstrated in Panel A, neither of these variables influence insulin binding in nonstimulated T-lympocytes. However, when 72 hour cultures of PHA-stimulated cells are incubated with ^{125}I-Insulin at either 15°C or 37°C, steady-state insulin binding is reached within 60 minutes. Although insulin binding for these cells is greater at 37°C than at 15°C, there is also considerable degradation at 37°C (data not shown). However, no detectable degradation is noted during the time course of binding at 15°C (data not

shown). For this reason, the effects of time and temperature on cell-associated degradation depicted by Panel B were examined following incubation of cells with ^{125}I-Insulin for 120 minutes at 15°C. As is illustrated in Panel B, there was a small amount of degradative activity at 15°C for stimulated cells but no degradation at either temperature in nonstimulated T-lymphocytes. However, at 37°C, degradative activity rapidly increased, reaching a plateau by 60 minutes of incubation. Furthermore, this degradation is cell-associated as no insulin degrading activity was detectable in the cell incubation media (data not shown).

The effects of insulin on PDH of PHA-stimulated and nonstimulated cells are summarized in Table 1. In these experiments, cells were preincubated with DL-Beta-hydroxybutyrate in order to reduce PDH_a. When insulin, at concentrations as low as 5.0 μU/ml (0.2 ng/ml), was added to the incubation media, PDH_a was enhanced in PHA-stimulated cells by 65%. At higher concentrations of insulin, the proportion of PDH_a in its active form increased from 35 to approximately 80%. As insulin did not affect PDH in nonstimulated cells, our data suggest that the development of insulin binding and/or degradation is a highly correlated phenomenon for postreceptor action of insulin on PDH.

TABLE 1

Insulin Action on the PDH of PHA-Stimulated and Nonstimulated Cultured T-lymphocytes of Man

Condition*	PDH_a (nmol $^{14}CO_2$/min/5x10^6 Cells)**	PDH	% PDH_a/PDH_t
Control	1.76±0.15	2.78±0.04	63
+Insulin (0.2 ng/ml)	1.62±0.03	2.99±0 03	54
+ Insulin (1.0 ng/ml)	1.78±0.08	2.83±0.06	62
+ Insulin (10.0 ng/ml)	1.83±0.01	2.91±0.01	63
PHA Control	1.08±0.05	3.11±0.10	35
+ Insulin (0.2ng/ml)	1.78±0.09	3.36±0.07	53
+ Insulin (1.0ng/ml)	2.59±0.11	3.01±0.13	86
+Insulin (10.0ng/ml)	2.53±0.05	3.19±0.07	79

* 72-hr. cultured cells incubated for 30 minutes at 37°C with 20mM DL-Beta Hydroxybutyrate and under the conditions described.
** Values represent the Mean ± SEM of 4 determinations.

DISCUSSION

PHA-stimulated T-lymphocytes have been studied in experimental animals and to a limited extent in man under conditions whereby insulin receptor generation has been correlated to physiologic insulin-induced responses, i.e. glucose uptake and oxidation (4,7). In the present studies, we report a hitherto unknown temporal relationship that appears to exist between the development of the insulin receptor in PHA-stimulated T-lymphocytes of man and the appearance of insulin degradative activity and PDH activation. Although no causal relationship could be induced from these studies, the concomitant presence of these three events is highly suggestive that binding and degradation are related to insulin action as these phenomena are evident in PHA stimulated but not in nonPHA-stimulated cells.

In summary, we have shown that activated T-lymphocytes provide a readily accessible tissue for studying insulin binding, degradation, and PDH response to insulin in man. The detailed studies on the localization of insulin degrading activity, its relationship to binding as well as the significance of these phenomena to overall insulin action in T-lymphocytes of normal and insulin resistant states require further investigation.

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