Mechanism of Glucocorticoid-induced Increase in Insulin Receptors of Cultured Human Lymphocytes*

I. George Fantus†, George A. Saviolakis, José A. Hedo, and Phillip Gorden§

From the Diabetes Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

Glucocorticoids increase the number of insulin receptors in cultured human lymphocytes. This effect is specific for the insulin receptor as growth hormone receptor concentration decreases. The effect is time-dependent, dose-dependent over the physiologic concentration range of glucocorticoid, reversible, and appears to be mediated via specific glucocorticoid receptors. Protein synthesis and glycosylation are required as the effect is inhibited by actinomycin D, puromycin, cycloheximide, and tunicamycin.

Isolated solubilized plasma membranes, as well as solubilized whole cells from glucocorticoid-treated lymphocytes, show the same increase in insulin receptors as do intact cells. The glucocorticoid-induced receptors are immunologically indistinguishable from control when precipitated by anti-receptor antibody. Glucocorticoids do not stabilize existing receptors since the degradation rate of receptors is actually increased. Regulation of the insulin receptor by insulin is independent of the steroid effect. However, there is a rightward shift of the dose-response curve for "down regulation" by insulin in the presence of glucocorticoid.

We conclude that glucocorticoids increase the number of insulin receptors in cultured human lymphocytes by increasing the synthesis of new receptors. This represents the first induction of insulin receptor synthesis by a pharmacologic agent.

Regulation of hormone receptors by heterologous hormones has been described for a number of different hormone receptor systems (1-5). Glucocorticoids in particular have been shown to modulate the affinity and/or receptor concentrations of a variety of hormone receptors (6-14). Physiologic concentrations of glucocorticoid increase the affinity of epidermal growth factor receptor for epidermal growth factor in a dose-dependent manner. This has been shown to be a specific effect which requires protein synthesis (11). It has recently been shown that glucocorticoids increase β-adrenergic receptors in human lung (14), inhibit the expression of Fc receptors on a human granulocyte cell line HL-60 (12), and inhibit the disappearance of 1,25-dihydroxycholecalciferol binding in cytosol from fetal rat calvaria (13).

The effects of glucocorticoids on the insulin receptor are variable depending on the cell type and conditions used in the study. Decreased affinity, as well as decreased number of receptors, has been reported in adipocytes after 48 h to 1 week of exposure to glucocorticoid (6, 7, 14-16). We (8) and others (9) have shown that exposure of cultured human lymphocytes to glucocorticoids in physiologic concentrations for 18 h at 37 °C results in a dose-dependent increase in the number of insulin receptors per cell. This in vitro model system has been useful in disclosing the role of insulin itself in the regulation of the insulin receptor (17). We have, therefore, examined in detail the mechanism of regulation of the insulin receptor by glucocorticoids in cultured human lymphocytes. We provide strong evidence for the requirement of new protein synthesis. In addition, we have employed this model to study the interaction of glucocorticoid and insulin in the regulation of the insulin receptor.

MATERIALS AND METHODS

Hydrocortisone, prednisolone, dexamethasone, aldosterone, and corticosterone (17-hydroxy-11-deoxycorticosterone) were purchased from Sigma. Cycloheximide, puromycin, and actinomycin D were also purchased from Sigma. Tunicamycin was a kind gift from Dr. M. Kauga of the Diabetes Branch, NIH.

Cell Cultures—Cultured human lymphocytes (IM-9 line) were grown as previously described (17). Lymphocytes in the late log phase of growth were centrifuged for 5 min at 1000 rpm and resuspended in fresh medium (RPMI 1640 with 25 mM Hepes7). The various steroids were dissolved in 70% ethanol and added to the culture media to yield final concentrations as described. An equivalent volume of 70% ethanol (diluent) was added to control cultures.

Insulin Binding—After exposure for a period of time (18 h unless otherwise specified) at 37 °C, the cells were sedimented for 5 min at 600 × g and resuspended in 50 mM Hepes lymphocyte buffer (pH 7.8) to a concentration of 3-8 × 10⁶ cells/ml. In those experiments in which insulin was added to the preincubation, the cells were washed three times with 1.0 M phosphate buffer (pH 7.4) with 1 mg/ml of bovine serum albumin for a total time of 60 min as previously described before resuspension in the Hepes buffer. There is no significant insulin remaining on the cells at the end of the wash (18).

[125]Iodoinsulin (porcine) was prepared at a specific activity of 100-150 µCi/µg by a modification of the chloramine-T method and was separated from free iodide by chromatography on a cellulose column (19). [125]I-insulin, 0.1 ng/ml, was incubated with the lymphocytes (3-8 × 10⁶ cells/ml) in the presence and absence of unlabeled insulin over a range of insulin concentrations from 0.1 through 10,000 ng/ml with a final incubation volume of 0.5 ml per assay tube. In these experiments, the incubation was carried out in 50 mM Hepes lymphocyte buffer (pH 7.8) for 90 min at 15 °C. Replicate 200-µl aliquots of the incubation mixture were transferred onto 200 µl of ice-cold buffer in microfuge tubes and centrifuged for 90 s. The supernatant was aspirated, the cell pellet contained in the tip of the microfuge tube was excised, and the radioactivity was counted. Non-specific binding, defined as the amount of [125]I-insulin bound to the cell pellet in the presence of 10⁶ ng/ml of insulin, was ≤1.5% of the total radioactivity. The pH remained constant throughout the incubation. Cell viability assayed by trypan blue exclusion was ≥90% unless otherwise specified. Specific binding is normalized to 10⁶ cells/ml.

1 The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Induction of the Insulin Receptor by Glucocorticoids

Solubilization of Cells and Plasma Membrane Receptors—Plasma membranes were isolated from the cultured human lymphocytes through step 1 of the procedure of Jett et al. (20). Briefly, cells were washed with RPMI 1640 medium and lysed with glycerol (90% w/v in the same medium). Pelleted cells were resuspended vigorously in 10 mM Tris/HCl, pH 7.4, containing 1.0 mM MgCl₂ and 1.0 mM CaCl₂ at 4 °C. The cell lysate was centrifuged at 700 × g to remove nuclei and at 3000 × g to remove mitochondria. The supernatant solution was layered over a cushion of 38% sucrose and centrifuged at 95,000 × g (Sorvall ultracentrifuge with AH 627 swinging bucket rotor) for 1.5 h at 4 °C. The interface band was aspirated, resuspended 3 times in 50 volumes of 50 mM Heps, 150 mM NaCl, pH 7.8, and pelleted by centrifugation at 45,000 × g for 45 min at 4 °C. The final pellet was resuspended in 50 mM Heps, 150 mM NaCl, pH 7.8, containing 1% Triton X-100 incubated for 45 min at 25 °C and centrifuged at 107,000 × g for 90 min at 4 °C. The supernatant, which contained the solubilized membranes, was used for the ¹²⁵I-insulin binding studies (21).

For the solubilization of whole cells, the cultured cells are mixed in 10 volumes of 50 mM Heps, 150 mM NaCl, pH 7.8, containing 1% Triton X-100 and incubated at 24 °C for 45 min. Unbroken cells and the debris are removed by centrifugation at 3300 × g and the supernatant was subsequently centrifuged at 107,000 × g for 90 min at 4 °C. The final supernatant which contained the whole cell-solubilized membranes was used for the insulin binding studies.

Immunoprecipitation of the Insulin Receptor—Immunoprecipitation of the insulin receptor is performed by the method of Harrison et al. (22). The anti-receptor antibody was obtained from serum of a patient with the Type B syndrome of insulin resistance (23). This antibody has previously been shown to precipitate the insulin receptor from several different tissues.

Measurement of the Degradation Rate of the Insulin Receptor—Following incubations in the presence and absence of hydrocortisone, the cell surface was iodinated by the lactoperoxidase method and solubilized. The solubilized radioactivity was immunoprecipitated by anti-insulin receptor antibody. The immunoprecipitated radioactivity was then applied to reducing gels of NaDodSO₄/polyacrylamide (7.5%) and electrophoresed as previously described (24).

RESULTS

Effects of Corticosteroid Dose and Specificity for the Glucocorticoid Receptor on Insulin Binding—When cultured human lymphocytes are exposed, over the physiologic concentration range, to different corticosteroids, there is a dose-dependent increase in insulin binding (Fig. 1). The potency of the interaction studied is dexamethasone > hydrocortisone > aldosterone > cortexolone. These doses and relative potencies are the same as reported for these steroids for other specific physiologic effects and corresponds to the relative affinities of these steroids for the glucocorticoid receptor (25). Estrogen and testosterone had no effects (data not shown). Further, we and others have previously shown that, under identical conditions, the binding of ¹²⁵I-human growth hormone in these cells is decreased (8, 9). Thus, the increased binding is a specific effect on the insulin receptor.

When the competition studies are analyzed by the method of Scatchard, assuming a negative cooperative model (26) for the analysis of the curvilinear plot, the increased binding can be accounted for by an increase in the number of binding sites (R₀) (8). Thus, a plot of the bound/free versus R₀ for the different steroids studied yields a strongly positive correlation (r = 0.75, p < 0.001) (Fig. 2). Briefly, in contrast, no consistent changes in receptor affinity were observed.

To further demonstrate the role of the glucocorticoid receptor in mediating the increased insulin binding, we employed the analogue cortexolone (17-hydroxy-11-deoxy cortisolone). This analogue has about one-half the potency of hydrocortisone at 20 times the dose of hydrocortisone. Cortexolone, while having a weak agonistic effect, is able to completely antagonize the effect of hydrocortisone in increasing insulin binding (Fig. 3). These data are consistent with previous studies that have demonstrated glucocorticoid receptors in the IM-9 cultured human lymphocyte.²

Time Course—In addition to dose dependence, the effect of glucocorticoids on the insulin receptor of the cultured human lymphocyte is also time-dependent; 18-20 h are required for the maximal effect, while at least 6 h is required for 10% of the effect (data not shown).

Reversibility—The increased binding induced by exposure to the steroids is not fully reversed by washing the cells with hormone-free medium (data not shown). This suggests that the binding of insulin is altered at the receptor site, consistent with the concept that the glucocorticoid receptor is involved in the event.²

² M. E. Lippman, personal communication.
Induction of the Insulin Receptor by Glucocorticoids

8279

CONTROL

0

HYDROCORTISONE 1.4 x 10^{-6} M

A CORTEXOLONE 1.29 x 10^{-6} M

INSULIN (ng/ml)

PERCENT 125I INSULIN BOUND

0

10

20

30

40

50

1

10

100

1000

INSULIN (ng/ml)

FIG. 3. Inhibition of glucocorticoid effect on insulin binding by cortexolone (17-hydroxy-11-deoxycorticosterone) in cultured human lymphocytes. Cells were preincubated for 18 h at 37 °C with hydrocortisone (1.4 x 10^{-6} M) alone, a 20-fold higher concentration of cortexolone (2.9 x 10^{-6} M) alone, or both. The cells were sedimented and washed, and insulin binding was performed as described. A, competition curves. Specific 125I-insulin bound is plotted versus insulin concentration. B, Scatchard plots. Bound/Free the ratio of bound to free hormone is plotted as a function of the total insulin bound, both normalized to 10 x 10^6 cells/ml. The intercept on the abscissa is representative of R0 (receptors/cell). (This is representative of three separate experiments.)

to a maximally effective concentration of glucocorticoid for 18-48 h is completely reversible within 48 h after washing the cells free of either dexamethasone or hydrocortisone (data not shown).

Effect of Metabolic Inhibitors on Corticosteroid-induced Increased Insulin Binding—When the cultured lymphocytes were preincubated for 18 h with a maximally effective concentration of hydrocortisone (0.5 μg/ml), the expected increase in insulin binding occurred (Fig. 4). However, when cycloheximide (10^{-6} M) was co-incubated with hydrocortisone, the steroid effect was completely blocked. Cycloheximide alone had no specific effect. Under these conditions of cycloheximide treatment, protein synthesis is inhibited by 85-90% (27). Cell viability in the presence of cycloheximide was decreased by 3-8% as compared to control cells.

Similar effects were seen using tunicamycin (0.5 μg/ml), an antibiotic that inhibits N-glycosylation of proteins during post-translational modification (28). Tunicamycin completely inhibits the increase in receptor number induced by hydrocortisone (Fig. 5). Note also that tunicamycin produces a 30% decrease in insulin binding as compared to control incubations (Fig. 5). Cell viability was decreased 15-20%. These concentrations of tunicamycin produce a 50-60% decrease in N-acetylglucosamine incorporation, but no change in protein synthesis (29). Since, however, we have not studied directly the effect of tunicamycin on protein synthesis, we cannot

FIG. 4. Inhibition of glucocorticoid effect on insulin binding by cycloheximide in cultured human lymphocytes. Cells were preincubated with diluent alone (control, A), hydrocortisone (1.4 x 10^{-6} M) alone (○—○), cycloheximide (10^{-6} M) alone (△—△), or both hydrocortisone and cycloheximide (■—■) for 18 h at 37 °C following which insulin binding was performed as described. A, competition curves; B, Scatchard plots. The data are representative of three separate experiments.
Induction of the Insulin Receptor by Glucocorticoids

exclude the possibility that the tunicamycin effect may in part be due to inhibition of protein synthesis.

Similar experiments (determining only total and nonspecific binding) were carried out using the protein synthesis inhibitor puromycin (30) and RNA synthesis inhibitor actinomycin D (31). After 18 h of preincubation with puromycin (1 μg/ml) or actinomycin (0.02 μg/ml), there was an approximately 65% and 25% inhibition of the effect of hydrocortisone on insulin binding, respectively (data not shown). These reagents produced a 15–20% decrease in cell viability after 18 h of incubation.

Effect of Glucocorticoid on Solubilized Receptor—A possible mechanism of action of glucocorticoids to increase insulin binding is the unmasking of insulin receptors already present but hidden within the plasma membrane or cytoplasm of the cell. Lymphocyte cell membranes were isolated and solubilized and insulin binding was measured. The solubilized membranes from glucocorticoid-treated cells demonstrated an increase in insulin binding compared to control membranes to the same degree as the intact cells (Fig. 6). This was due to an increase in the number of insulin receptors/mg of membrane protein. Furthermore, solubilized whole cell preparations from glucocorticoid-treated cells also demonstrated the same degree of increased insulin binding (data not shown).

Immunoprecipitation with Anti-insulin Receptor Antibody—To further evaluate the nature of the glucocorticoid-induced receptor, solubilized receptor from control and glucocorticoid-treated cells were precipitated with anti-insulin receptor antibody from the serum of a patient with the syndrome of Type B insulin resistance (23). These antibodies precipitated each preparation in an indistinguishable fashion, indicating the appearance of immunologically intact receptor (Fig. 7).

Effect of Glucocorticoid on the Degradation Rate of the Insulin Receptor—An increase in the number of insulin receptors induced by glucocorticoids in intact cells, solubilized plasma membranes, or solubilized whole cells could result from the synthesis of new receptors or stabilization of existing receptors. To distinguish these two effects, we have studied
Induction of the Insulin Receptor by Glucocorticoids

8281
directly the degradation rate of receptors in control and glu-
cocorticoid-treated cells by a newly described methodology
(24).

Cells were incubated without or with glucocorticoid until
insulin receptors were maximally expressed (see Fig. 1 of Ref.
8). The control and glucocorticoid-treated cells were then
iodinated and the batch previously treated with glucocorticoid
was re-exposed to the steroid for an additional 6 h. At 0, 2, 4,
and 6 h, an aliquot of cells was solubilized, the radioactivity
was precipitated with anti-insulin receptor antibody, and ap-
plied to and electrophoresed on a reducing sodium dodecyl
sulfate gel. The receptor antibody complexes were separated by centrifugation at
10,000 × g for 5 min at 4 °C. Maximum precipitation was achieved
with a 1:100 serum dilution and designated 100%. The per cent of
maximal 125I-insulin receptor precipitated is plotted as a function of
decreasing antibody concentration (increasing dilution) for control
and glucocorticoid-treated membrane receptors.

Fig. 7. Immunoprecipitation of 125I-insulin-labeled solubi-
lized lymphocyte membranes by anti-insulin receptor anti-
body. After preincubation of cells with diluent (C=O) or hydro-
cortisone (1.4 × 10^{-5} M) (O—O) and preparation of solubilized
membranes, the control and glucocorticoid-treated membranes were
labeled with 125I-insulin for 1 h at 24 °C. These preparations were
then incubated with anti-insulin receptor antibody or control serum
at 4 °C for 8 h. Second antibody (sheep anti-human globulin) was
then added and incubation was continued for 16 h at 4 °C. Labeled
receptor antibody complexes were separated by centrifugation at
10,000 × g for 5 min at 4 °C. Maximum precipitation was achieved
with a 1:100 serum dilution and designated 100%. The per cent of
maximal 125I-insulin receptor precipitated is plotted as a function of
decreasing antibody concentration (increasing dilution) for control
and glucocorticoid-treated membrane receptors.

Fig. 8. Effect of hydrocortisone on insulin receptor turnover
rate in cultured human lymphocytes. Cells were preincubated with
or without hydrocortisone (1.4 × 10^{-5} M) for 18 h at 37 °C. Rates
of degradation of iodinated insulin receptor subunits in the presence
(C=O) and in the absence (O—O) of hydrocortisone. Following
the preincubation with hydrocortisone, cells were iodinated with
lactoperoxidase and Na^{125I} as previously described (24) and returned
to culture with and without hydrocortisone. Cell viability, as mea-
sured by dye exclusion, was greater than 95% throughout the experi-
mental period after the lactoperoxidase iodination. At the times
indicated in the figure, equal volumes of cells were solubilized in
Trition X-100 and the insulin receptors were immunoprecipitated with
anti-receptor serum (B-2). The immunoprecipitates were analyzed by
NaDodSO_{4}/polyacrylamide gel (7.5% electrophoresis and autoradi-
ography. Quantitative estimates of the radioactivity in the receptor
subunits were obtained by cutting the gels and counting the radioac-
tivity in a γ counter. Half-lives were obtained by extrapolation and
are given in hours.

Fig. 9. Interaction of insulin and glucocorticoid on insulin
receptor regulation in cultured human lymphocytes. Cells were
preincubated with hydrocortisone (1.4 × 10^{-5} M) and insulin
(0—O) or diluent and insulin (C=O) at concentrations indicated
for 18 h at 37 °C. The cells were extensively washed as described to
remove bound insulin and 125I-insulin binding was performed. Per-
cent 125I-insulin specifically bound at tracer concentrations are plotted
versus the insulin concentrations in the preincubation. Data are the
mean ± S.E. of three separate experiments.

Interaction of Insulin and Glucocorticoid on Receptor
Regulation—Cultured human lymphocytes preincubated
with insulin show a time-, temperature-, and insulin concen-
tration-dependent "down regulation" of the number of insulin
receptors (17). Hydrocortisone exposure (above) results in an
opposite effect. The regulatory effects of each hormone can
be demonstrated in the presence of both together. Thus, cells
exposed to both insulin and hydrocortisone demonstrate
greater insulin binding than those exposed to the same con-
centration of insulin alone. As well, they demonstrate less
insulin binding when compared with cells exposed to a similar
concentration of hydrocortisone alone (Fig. 9). In order to
detect any influence of glucocorticoid on down regulation by
insulin, cells in the presence and absence of a maximally
effective concentration of hydrocortisone were exposed to
increasing concentrations of insulin for 18 h. The relative
decrease in insulin binding by preincubation with insulin was
partially inhibited by glucocorticoid. The inhibitory effect was
more apparent with increasing concentrations of insulin and
greater degree of down regulation. Thus, there appears to be
a significant shift of the "dose-response" curve for insulin
receptor down regulation by insulin in the presence of hydro-
cortisone (Fig. 10).
**Induction of the Insulin Receptor by Glucocorticoids**

**Fig. 10. Interaction of insulin and glucocorticoid on insulin receptor regulation in cultured human lymphocytes.** Protocol as in Fig. 9. **[Graph]**

We have shown that glucocorticoids specifically increase the number of insulin receptors on cultured human lymphocytes in a time- and dose-dependent manner. The affinity of the insulin receptor remains unaltered. The relative potencies indicate a specific glucocorticoid hormone effect. This is supported by the lack of effect of the sex steroid, estrogen, and very weak effect of the mineralocorticoid, aldosterone. The specificity is further established by the demonstration of a decrease in growth hormone binding. Reversibility of removal of the glucocorticoids indicates dependence on the continued presence of the hormone.

The relative potencies of the glucocorticoids depends on their affinities for the glucocorticoid receptor. The potency series suggests that the effect on the insulin receptor is mediated via the interaction of glucocorticoid with its own receptor. This is confirmed by the demonstration that the effect can be inhibited by the competition antagonist corticosterone, which binds to the glucocorticoid receptor but has minimal intrinsic activity.

Glucocorticoid hormones have been shown to have physiological actions on multiple cellular processes. These may be direct alterations of intracellular enzyme systems (25) or indirect, so-called “permissive” actions (32, 33), which may involve regulation of heterologous hormone receptors (11, 14). Many actions of glucocorticoid which are manifested initially by binding of the hormone to its receptor have been found to require RNA and protein synthesis (34). The time course of the appearance of the increase in insulin receptor suggested that this might be the case since the first increase (10% of maximal) required at least 6 h while no effect was observed after 3 h of exposure. A requirement for protein synthesis has been demonstrated in the regulation of epidermal growth factor receptors by glucocorticoid (11).

Experiments with inhibitors of RNA (actinomycin D) and protein (puromycin, cycloheximide) synthesis provide further strong evidence that new protein synthesis is required for this effect. As well, the inhibition of post-translational protein glycosylation by tunicamycin suggests that the new protein synthesized is a glycoprotein. The insulin receptor is known to be a glycoprotein and it appears that new receptor synthesis is induced by glucocorticoids. A second possibility is the synthesis of an inhibitor of receptor degradation. If this were the case, however, the turnover rate of the receptor would be slowed down. Fig. 8 shows clearly that this is not the case; if anything, the glucocorticoid increases the rate of receptor turnover. A third less likely possibility is the unmasking by glucocorticoid of cryptic membrane receptor. However, solubilized lymphocyte membranes from glucocorticoid-treated cells demonstrate a similar increase in insulin receptor number. Furthermore, glucocorticoid-treated solubilized whole cells also demonstrate the same increase in insulin receptor numbers as the membranes and intact whole cells.

The “new” membrane insulin receptors are indistinguishable immunologically from normal insulin receptors as assessed by anti-receptor antibody binding and have the same subunit structure.

Recent evidence indicates that this effect of glucocorticoids on insulin receptors is not confined to cultured human lymphocytes; a similar effect has been found in cultured human fibroblasts. Other cells, however, such as adipocytes (15) and 3T3-L1 fatty fibroblasts (16) have been found to have decreased insulin binding after exposure to glucocorticoids. These variable results suggest that not all tissues are regulated in the same way with regard to insulin receptors. Thus, extrapolation from changes observed in one tissue to all tissues may not always hold true. This should be noted in the case of monocytes and red blood cell studies currently carried out in many laboratories and assumed to represent the state of the receptor on target tissues (8, 35).

The interactions of two regulators of receptor function have not previously been studied in detail. When compared with exposure to insulin alone, cells exposed to both insulin and hydrocortisone have higher binding. Thus, the glucocorticoid effect is observed in the presence of “down regulation” of receptor by insulin. As well, down regulation clearly occurs in the presence of glucocorticoids. This is similar to the combined effects of dexamethasone and epidermal growth factor on epidermal growth factor binding in cultured fibroblasts (11). When the dose-response relationship of down regulation of insulin receptor is examined there is a right-ward shift in the presence of hydrocortisone indicating resistance to down regulation by insulin.

We, therefore, conclude that glucocorticoids specifically increase the number of normal intact insulin receptors in cultured human lymphocytes by inducing new receptor synthesis. If this explanation of our data is correct, it represents the first induction of the insulin receptor by a pharmacologic agent.

**Acknowledgments**—We would like to thank Janice Ryan and Maxine A. Lesniak for help with the cultured cells, Drs. Jesse Roth, Carl Grunfeld, George L. King, and Emmanuel Van Obberghen for their helpful discussion, and Carol Culwell for excellent secretarial assistance.

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Induction of the Insulin Receptor by Glucocorticoids


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