Glucocorticoids increase insulin binding and the amount of insulin-receptor mRNA in human cultured lymphocytes

Yoshikazu SHIBASAKI,*§ Hiroshi SAKURA,* Masato ODAWARA,* Masabumi SHIBUYA,† Yasunori KANAZAWA,* Yasuo AKANUMA,‡ Fumimaro TAKAKU* and Masato KASUGA*

*Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan 113, †Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shiroganedai, Minato-ku Tokyo, Japan 108, and ‡Institute for Diabetes Care and Research, Asahi Life Foundation, 1-6-1 Marunouchi, Chiyoda-ku, Tokyo, Japan 100

The effect of steroid hormones on insulin binding and the amount of insulin-receptor mRNA was examined in IM-9 lymphocytes. Cortisol and cortexolone, but not oestrogen, increased both the binding of insulin and the amount of insulin-receptor mRNA in a time- and dose-dependent manner. Cortisol was most potent, and induced a 2-fold increase in insulin binding and a 4-fold increase in mRNA. The elevation in binding was due to an increased number of insulin receptors at the cell surface. The increase in mRNA involved all four of the insulin-receptor mRNAs and could not be inhibited by cycloheximide. The cortisol-induced increase in mRNA was associated with a 3–4-fold increase in the synthesis of pro-receptor. The relative potency of the three steroids indicated that these effects were mediated by an interaction with the glucocorticoid receptor. The results of this study suggest that cortisol can increase the number of insulin receptors at the cell surface by increasing the amounts of insulin-receptor mRNA and the synthesis *de novo* of insulin receptors.

INTRODUCTION

Since insulin initiates its effects by binding to insulin receptors, the concentration of the insulin receptor on the cell surface is important (Kahn, 1976). The number of insulin receptors on the surface of target cells changes in several physiological and pathological conditions (Bar & Roth, 1977). Glucocorticoids have been shown to increase the number of insulin receptors on the surface of cultured cells (Funtus *et al.*, 1982). According to biosynthetic labelling experiments, this increase is due to an enhanced rate of receptor biosynthesis, without change in the rate of receptor degradation (Rouiller *et al.*, 1985).

The cDNA for the human insulin receptor has been cloned (Ullrich *et al.*, 1985; Ebina *et al.*, 1985). Using the insulin-receptor cDNA, we were able to demonstrate that glucocorticoids increased both the number of insulin receptors at the surface of cultured human lymphocytes (IM-9) and the amounts of insulin-receptor mRNA.

MATERIALS AND METHODS

Cell cultures

Human lymphocytes (IM-9 line) (kindly provided by Dr. M. A. Lesniak, Diabetes Branch, N.I.H., Bethesda, MD, U.S.A.) were grown in RPMI-1640 medium containing 10% (v/v) fetal-calf serum. In the late-exponential phase of growth, the cells were transferred to new culture flasks containing 70 ml of the medium and incubated at 37 °C. After 24 h [$2(-3) \times 10^5$ cells/ml], cortisol, cortexolone or oestrogen was added to the media and the cultures were incubated at 37 °C as indicated. Subsequently, 40 ml portions of culture

suspension were used for the preparation of RNA, and 30 ml portions for the assay of insulin binding.

Insulin binding

The cells for insulin binding were sedimented for 5 min at 800 *g*, and resuspended in 50 mM-Hepes binding buffer (50 mM-Hepes, 120 mM-NaCl, 2 mM-MgSO₄, 1 mM-EDTA, 10 mM-glucose, 15 mM-sodium acetate and 10 mg of bovine serum albumin/ml, pH 8.0). A tracer amount (60 pM) of ¹²⁵I-insulin (sp. radioactivity 90–110 μCi/μg) (New England Nuclear, Boston, MA, U.S.A.) was incubated with the lymphocytes (5×10^6 cells/ml) in the presence or absence of unlabelled insulin over a range of concentrations from 0 to 10 μM in a final incubation volume of 0.25 ml. Insulin binding was performed for 3 h at 15 °C. The tubes were then centrifuged (10000 *g*) for 60 s and the supernatant aspirated as described previously (Kosmakos & Roth, 1980). The radioactivity of the pellet was counted.

Determination of insulin-receptor mRNA

Total RNA was prepared from 2×10^7 cells by the LiCl/urea procedure (Auffray & Rougeon, 1979). Then 20 μg of total RNA was electrophoresed on a 1%-formaldehyde/agarose gel, and RNAs were transferred to a nitrocellulose membrane (Schleicher and Schuell). A 2-kilobase (kb) *EcoRI/XhoI* fragment of plasmid pHIR13-1, encoding human insulin-receptor cDNA (Shaw & Bell, 1985) (kindly provided by Dr. G. I. Bell, University of Chicago), and a 0.7 kb *HindIII/HincII* fragment of human insulin-receptor genomic clone were used as probes. The latter clone was isolated from a human placental library with a synthetic oligonucleotide probe and found to be identical with part of the insulin-receptor cDNA (nucleotide nos. 147–700 in Ullrich *et al.*,

§ To whom reprint requests should be addressed.

1985). Hybridization was carried out at 65 °C overnight in a buffer containing 6 × SSC (1 × SSC = 0.15 M-NaCl/0.015 M-trisodium citrate), 5 × Denhardt's solution (100 × Denhardt = 2% bovine serum albumin, 2% polyvinylpyrrolidone, 2% Ficoll), 0.1% SDS, 100 µg of denatured salmon sperm DNA/ml and ³²P-labelled nick-translated probe DNA. The nitrocellulose membranes were washed quickly in 2 × SSC at room temperature and then three or four times for 30 min in 0.1% SDS/0.1 × SSC at 65 °C. Filters were autoradiographed with intensifying screens. For determination of the β-actin mRNA, the filter was hybridized to a ³²P-labelled nick-translated cDNA fragment from plasmid 91, encoding mouse actin cDNA (kindly provided by Dr. M. E. Buckingham, Institut Pasteur, Paris, France).

Receptor pulse-chase studies

IM-9 lymphocytes were grown in RPMI 1640 medium as described above. At the semi-confluent phase (0.4×10^6 cells/ml), the cells were divided equally into three new culture flasks each containing 350 ml of the medium, and incubated with 1.4 µM-cortisol for 0 (control), 2 and 8 h at 37 °C. After incubation the cells were quickly washed twice with 50 ml of phosphate-buffered saline (6.7 mM-sodium/potassium phosphate/0.85% NaCl, pH 7.4), incubated at 37 °C for 20 min (the pulse) in 10 ml of glucose-free RPMI 1640 medium containing 10% dialysed fetal-calf serum and 5 mCi of D-[2-³H(n)]mannose (sp. radioactivity 20–30 Ci/mmol; New England Nuclear). The cells were then washed twice with 50 ml of phosphate-buffered saline and resuspended in 50 ml of RPMI 1640 medium containing 2 mM-mannose and 10% fetal-calf serum, and incubated at 37 °C for 30 min (the chase). Until the end of the chase, all the washing buffers and culture media contained 1.4 µM-cortisol, except for the control flask.

After the chase, the cells were washed three times with cold phosphate-buffered saline, collected by centrifugation and solubilized in 1 ml of 150 mM-NaCl/50 mM-Hepes (pH 7.4) containing 1% (v/v) Triton X-100, 2 mM-phenylmethanesulphonyl fluoride (Sigma) and 100 Kallikrein-Inactivating Units of aprotinin/ml, for 30 min on ice with continuous stirring. Soluble materials were separated by ultracentrifugation (200 000 g, 4 °C, 45 min) and the supernatants were pre-adsorbed with 150 µl of Pansorbin (Behring Diagnostics) for 2 h at 4 °C. After centrifugation (15 000 g), the supernatants were mixed with insulin-receptor autoantibodies obtained from a patient with insulin resistance and acanthosis nigricans (B-8) (kindly provided by Dr. C. R. Kahn, Joslin Diabetes Center, Boston, MA, U.S.A.) at a dilution of 1:40. After overnight incubation at 4 °C, the immune complexes were adsorbed with 150 µl of Pansorbin, washed three times with 50 mM-Hepes buffer (pH 7.4) containing 0.15 M-NaCl, 1% Triton X-100, 0.1% SDS and 1 mM-EDTA, and then washed with 50 mM-Hepes buffer (pH 7.4) containing 0.1% Triton X-100 and 0.1% SDS. The pellets were then boiled for 5 min in 100 µl of Laemmli (1970) buffer containing 100 mM-dithiothreitol.

Pansorbin was removed by centrifugation (15 000 g), and the supernatant was analysed by SDS/polyacrylamide-gel electrophoresis, with a 7%-polyacrylamide resolving gel. Gels were treated with EN³HANCE (New England Nuclear), dried, and autoradiographed for 20 days at –70 °C with an intensifying screen.

RESULTS

Time-dependent effects of cortisol on insulin binding and the amounts of insulin-receptor mRNA

We examined the time-dependent effects of cortisol on both ¹²⁵I-insulin binding and the amount of insulin-receptor mRNAs. Incubation of human cultured lymphocytes (IM-9 line) with cortisol (1.4 µM) increased ¹²⁵I-insulin binding to the cells in time-dependent fashion (Fig. 1). Thus ¹²⁵I-insulin binding increased by 15% after 2 h of incubation with cortisol, and by 85% after 20 h of incubation. This increase in ¹²⁵I-insulin binding was due to an increased receptor number rather than increased receptor affinity (results not shown).

From a separate sample of these cells, total cellular RNA was isolated, fractionated, bound to a nitrocellulose filter and hybridized to a ³²P-labelled nick-translated fragment of human insulin-receptor probe. Northern-

Fig. 1. Time-dependent effects of cortisol on ¹²⁵I-insulin binding to human cultured lymphocytes (IM-9 line)

The IM-9 lymphocytes were preincubated with 1.4 µM-cortisol at 37 °C for the indicated periods of time. After preincubation, the ¹²⁵I-insulin-binding assay was carried out as described in the Materials and methods section. The results represent means ± S.E.M. of three separate experiments.

Fig. 2. Time-dependent effect of cortisol on the amount of insulin-receptor mRNA

Total RNA (20 µg) from IM-9 lymphocytes previously incubated with 1.4 µM-cortisol for 0–20 h was electrophoresed on a formaldehyde/agarose gel, transferred to a nitrocellulose membrane and hybridized with ³²P-labelled fragments of insulin-receptor cDNA. All of the mRNAs specific for the insulin receptor were increased. Numbers below lanes indicate time (h) of incubation with cortisol.

blot hybridization revealed four bands, of 11.0, 9.5, 8.5 and 7.0 kb, in human lymphocyte (IM-9) mRNA (Fig. 2). These four bands probably represent the mRNA of human insulin receptors, as previously described (Ullrich *et al.*, 1985; Ebina *et al.*, 1985). As band patterns were identical with two probes, the insert of pHIR 13-1 was used in the following hybridizations. Densitometer examination indicated an increase in insulin-receptor mRNA within 1 h after addition of 1.4 μ M-cortisol; 2 h after this addition, insulin-receptor mRNA increased 2.5–3.5-fold, and at 20 h there were still further increases. The increase in the labelling after cortisol treatment occurred with all four bands of the insulin-receptor mRNA (Fig. 2).

Dose-dependent effects of cortisol

The dose-dependent effect of cortisol on both 125 I-insulin binding and the amount of insulin-receptor mRNAs was examined after incubation of lymphocytes with cortisol for 20 h. Cortisol was found to increase 125 I-insulin binding to these cells in a dose-dependent fashion (14 nM–1.4 μ M), with a half-maximal effect at 0.1 μ M (Fig. 3). Similarly, the amount of insulin-receptor mRNAs was also increased in a dose-dependent manner (Fig. 4). Furthermore, the dose/response curves for these two effects of cortisol were similar. Hence, by densitometric analysis, the concentration of cortisol for half-maximal effects at the level of insulin-receptor mRNA was also about 0.1 μ M. At the highest concentration (1.4 μ M), the intensity of 32 P-labelling of all four bands increased by more than 4-fold. As a control, the amount of β -actin was examined by Northern-blot hybridization by using 32 P-labelled nick-translated plasmid 91, specific for actin. Cortisol did not alter the 32 P-labelling of one major band of 2.8 kb representing mRNA for β -actin (results not shown).

Specificity of the effect of cortisol

To examine the role of the glucocorticoid receptor in these effects on the number of insulin receptors and amounts of mRNA, we examined the effect of oestrogen (17 β -oestradiol) and cortexolone (17-hydroxy-11-deoxy-

Fig. 3. Dose-dependent effects of steroids on 125 I-insulin binding

IM-9 lymphocytes were incubated in the presence of different concentrations of cortisol, cortexolone or 17 β -oestradiol for 20 h at 37 °C. The insulin-binding assay was carried out as described in the Materials and methods section.

Fig. 4. Dose-dependent effects of cortisol on the amount of insulin-receptor mRNA

Total RNA (20 μ g) taken from cells preincubated with different amounts of cortisol for 20 h was fractionated on a formaldehyde/agarose denaturing gel, transferred to a nitrocellulose membrane and hybridized with 32 P-labelled fragments of insulin-receptor cDNA as described in the Materials and methods section. Lane 1, control; lane 2, 14 nM-cortisol; lane 3, 0.14 μ M-cortisol; lane 4, 1.4 μ M-cortisol.

Fig. 5. Dose-dependent effects of oestrogen or cortexolone on the amount of insulin-receptor mRNA

Total RNA (20 μ g) from cells preincubated with steroid for 20 h was electrophoresed on formaldehyde gel, transferred and hybridized as described in the Materials and methods section. (a) Effect of oestrogen: lane 1, control; lane 2, 1.0 μ M-oestrogen; lane 3, 0.1 mM-oestrogen. (b) Effect of cortexolone: lane 1, control; lane 2, 0.29 μ M-cortexolone; lane 3, 2.9 μ M-cortexolone; lane 4, 29 μ M-cortexolone; lane 5, 1.4 μ M-cortisol.

corticosterone). Compared with cortisol, the latter has about one-fortieth of the affinity for glucocorticoid receptors (Munck, 1971). Incubation of IM-9 lymphocytes with oestrogen (100–1 μ M) had no effect on either 125 I-insulin binding (Fig. 3) or the amounts of insulin-receptor mRNAs (Fig. 5a).

In contrast, after incubation of IM-9 lymphocytes with cortexolone, 125 I-insulin binding to these cells increased, although the dose/response curve was shifted to the right (Fig. 3). The concentration of cortexolone for a half-maximal increase in insulin binding was about 5 μ M. Insulin-receptor mRNA amounts were also increased in a dose-dependent fashion by cortexolone;

Fig. 6. Effect of cycloheximide on amounts of insulin-receptor mRNA

IM-9 lymphocytes were incubated in the absence or presence of cycloheximide or/and cortisol at 37 °C. After 20 h incubation, total RNA was prepared from the lymphocytes, and the amounts of insulin-receptor mRNA were evaluated as described in the Materials and methods section. Lane 1, control; lane 2, 1.4 μ M-cortisol; lane 3, 1.4 μ M-cortisol plus 10 μ M-cycloheximide.

however, the amount induced by 29 μ M-cortexolone was less than that induced by 1.4 μ M-cortisol (Fig. 5b). The relative potencies of these three steroids for increasing either insulin binding or the amounts of insulin-receptor mRNA parallel their relative affinities for the glucocorticoid receptor. This observation suggests that these steroid effects may have been mediated by glucocorticoid receptors.

Effect of cycloheximide

To distinguish between a primary response, which does not require protein synthesis, and a secondary response, which needs protein synthesis, we performed experiments with cycloheximide, an inhibitor of mRNA translation into protein. When cycloheximide (10 μ M) was added together with cortisol (1.4 μ M) to the culture media and the IM-9 lymphocytes were then incubated for 20 h, 125 I-insulin binding was not increased. However, the amount of insulin-receptor mRNA was increased to almost the same value as that obtained in the presence of only cortisol (Fig. 6, lanes 2 and 3). Incubation of the lymphocytes with cycloheximide for 12 h before the treatment with cortisol revealed similar results (not shown). These findings suggest that the increase in insulin-receptor mRNA is a primary response. They also indicate that the increase in insulin binding after cortisol treatment requires the synthesis *de novo* of new insulin receptors.

Pulse-chase labelling of pro-receptor of insulin receptors

To investigate the relationship between the amount of insulin-receptor mRNA and the number of insulin receptors on the cell surface, we examined the synthesis of insulin-receptor proteins. Hence, human cultured lymphocytes (1.4×10^8 cells) were incubated with 1.4 μ M-

Fig. 7. Pulse-chase labelling with [3 H]mannose of the insulin-receptor precursor and α -subunit in cultured human lymphocytes (IM-9 line) in the absence or presence of 1.4 μ M-cortisol

Cells were incubated for 0, 2 or 8 h with 1.4 μ M-cortisol. Then cells were pulse-labelled for 20 min in glucose-free medium containing [3 H]mannose and subsequently chased in medium containing 2 mM unlabelled mannose for 30 min at 37 °C. After solubilizing the cells, their insulin receptors were immunoprecipitated with anti-insulin-receptor antibodies and analysed by electrophoresis and autoradiography as described in the Materials and methods section.

cortisol for 0, 2 or 8 h. The cells were then labelled with [3 H]mannose for 20 min at 37 °C (the pulse), followed by incubation with 2 mM-mannose for 30 min at 37 °C (the chase). Since we were interested in the rate of synthesis of newly synthesized insulin receptor, we used a 30 min chase (Rouiller *et al.*, 1985). The cells were then solubilized, immunoprecipitated with anti-insulin-receptor antibodies and analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography.

The treatment with cortisol resulted in increased labelling of a major band of M_r 190 000. This is probably the precursor of the insulin receptor, as described by Hedo *et al.* (1983). Another band, of M_r 135 000, was detected. This is presumably the α -subunit of insulin receptor (Kasuga *et al.*, 1982). As most of the labelled precursor of insulin receptors had not matured after only a 30 min chase period, the α -subunit appeared as only a faint band (Hedo *et al.*, 1983; Rouiller *et al.*, 1985). The incubation with cortisol for 2 or 8 h increased the labelling of the M_r -190 000 band 3–4-fold as compared with the control, by densitometric analysis of the autoradiogram (Fig. 7). These results suggested that the rate of synthesis of insulin-receptor precursor increased 3–4-fold. This finding agrees closely with the effects of cortisol on the amount of insulin-receptor mRNA (Fig. 2).

DISCUSSION

We have demonstrated that cortisol specifically increases the amounts of insulin-receptor mRNA in a cultured human lymphoid cell line (IM-9) in a dose- and time-dependent manner. Glucocorticoids have previously been shown to increase the number of insulin receptors at the surface of IM-9 lymphocytes (Funtus *et al.*, 1982), presumably owing to an increase in receptor biosynthesis (Rouiller *et al.*, 1985). In this study, we confirm that cortisol increases the number of insulin receptors at the surface of IM-9 lymphocytes. In addition, we found that incubation with cortisol increases the amount of insulin-receptor mRNA and the rate of synthesis of insulin-receptor precursor. The time-course and dose/response studies, and the dose/response comparison of cortisol and cortexolone, suggest that the increase in the amount of insulin-receptor mRNA can account for the increased number of insulin receptors at the cell surface of IM-9 lymphocytes.

However, when evaluated quantitatively, these results show that about a 4-fold increase (3.8 ± 0.2 ; mean $\pm$ S.D., $n = 4$) in the amount of insulin-receptor mRNA led to less than a 2-fold increase (1.8 ± 0.4 ; mean $\pm$ S.D., $n = 3$) in receptors at the cell surface. This discrepancy is statistically significant by Student's *t* test ($P < 0.05$). To investigate this discrepancy, the rate of synthesis of the insulin receptor precursor was examined. Pulse-chase labelling revealed that cortisol treatment increased the amount of newly synthesized proreceptor 3–4-fold. Therefore the increased amount of insulin-receptor mRNA correlated well with the amount of newly synthesized receptor. In addition, when cells were incubated with cortisol in the presence of cycloheximide, there was no increase in insulin binding. This indicates that the increase in binding seen in the dose/ and time/response experiments probably involved the insertion of newly synthesized insulin receptors into the plasma membrane. Regulating the rate of insertion of newly synthesized insulin receptors, along with the rate of receptor internalization and recycling (Fehlmann *et al.*, 1982), provides cells with a rapid control of receptor numbers at the cell surface. At present this mechanism is incompletely understood. However, the existence of other steps for regulating cell-surface receptors may explain the discrepancy found in this study between the effects of cortisol on the number of insulin receptors at the cell surface and on the amounts of insulin-receptor mRNAs.

Increased amounts of insulin-receptor mRNA can be due to changes in mRNA synthesis, stability, processing or transport. The relative potencies of the three steroids correlated closely with their affinity for the glucocorticoid receptor. This suggests that their effect on the amount of insulin-receptor mRNA was probably mediated through

an interaction with this receptor. Others have reported that the receptors for steroid hormones can bind to specific sites on the genome and regulate the transcription of specific genes (Yamamoto, 1985). Cortisol increased the amount of insulin-receptor mRNA without requiring protein synthesis. It is possible that this effect may have been due to a decreased rate of insulin-receptor mRNA degradation, or to an increased rate of insulin-receptor mRNA transcription. This raises the possibility that the glucocorticoid receptor may bind to regulatory sites on the insulin-receptor gene. Such a possibility indicates that an analysis of the upstream region of insulin-receptor gene would be very interesting.

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