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EFFECTS OF GLUCONEOGENIC HORMONES ON INSULIN BINDING IN INTACT HUMAN RED BLOOD CELLS

LEBOGANG MOKOENA, MARIANE ALBERTS and, ERROL TYOBEKA

Departments of Biochemistry and Medical Laboratory Sciences,
University of the North,
Private Bag, X1106 SOVENGA, 0727
REPUBLIC OF SOUTH AFRICA

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The effects of gluconeogenic hormones, adrenaline and cortisol, on insulin binding were studied in intact human red blood cells. Insulin binding was significantly decreased when red blood cells were preincubated with 1.0 $ug \cdot ml^{-1}$ adrenaline or cortisol respectively. The Scatchard plot suggested that this was due to a decrease in surface receptor concentration. Furthermore, it showed that adrenaline also increased insulin receptor affinity. The negative co-operativity affinity profile demonstrated that adrenaline caused a rise in only the upper limit average affinity, K_i, of the insulin receptor.

The insulin receptor is a dynamic transmembranous glycoprotein localized primarily in the cell membrane of almost all mammalian cells. Research has shown that this receptor is an oligomer constituted of two pairs of subunits, i.e. 2α-subunits (M_r = 135 000) and 2ß-subunits (M_r = 95 000), (1-6).The primary event of insulin action has been shown to be its binding to this receptor on the plasma membrane of target cells (7). After its formation, the insulin-receptor complex is immediately internalized (8-16). The internalized receptors are then either degraded by lysosomal enzymes or recycled back to the membrane (8-10,

ABBREVIATIONS:
Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; tris, tris (hydroxymethyl) aminomethane; EDTA, ethylenediaminetetra-acetic acid.

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12, 13, 17-19). Some studies (20-22) have provided evidence indicating that cells which were initially exposed to high levels of a ligand (insulin) show a decrease in receptor binding on subsequent exposure. This ligand induced reduction in cell responsiveness is referred to as down-regulation. It has been proposed that internalization of the receptor might be the initial step in down-regulation (8 - 10). Indeed, Kasuga et al. (23) and Knutson et al. (24) even suggest that down-regulation is a consequence of degradation of the internalized insulin receptor complex by lysosomal enzymes.

The effects of hormones on the insulin receptor and insulin responsiveness have received considerable attention. Research in this field is continuing despite the observed contradiction of results. In vivo administration of steroids has resulted in decreased (25-27), increased (28) or unmodified (27, 29, 30) insulin binding in adipocyte (25, 29), erythrocyte (26, 30), hepatocyte (27) or monocyte (25, 28, 30) systems. In a recent report by Müller et al. (31) it was suggested that hyperthyroidism can reduce insulin receptor binding in monocytes and cultured mitogen-stimulated T-lymphocytes, but has no effect on insulin binding to red blood cells. Since there are discrepancies and contradictions in studies on the effects of hormones on insulin binding, further investigation of this matter seems necessary. In the present study the effect of gluconeogenic hormones, adrenaline and cortisol, on insulin binding in erythrocytes was investigated. Our findings show that gluconeogenic hormones cause a decrease in receptor concentration on the cell membrane.

MATERIALS AND METHODS

REAGENTS AND SOLUTIONS

Mono ^{125}I-(Tyr A14) human insulin (specific activity = 2 000 Ci/mmol) was obtained from Amersham International, U.K.; unlabelled porcine insulin and cortisol from Sigma Chemicals, USA; adrenaline from Fluka, A.G., Switzerland; and dibutyl phthalate from Riedel-de Haën, Hannover. All the reagents used were of the highest purity available. The composition of the buffer G was as follows (in mM) : Hepes, 50; tris, 50; $MgCl_2.6H_2O$, 10; EDTA, 2; glucose, 10; $CaCl_2$, 10; NaCl, 50; and KCl, 5,

containing 0,1% bovine serum albumin. The pH was adjusted to 6 or 8 using a weak solution of HCl.

SUBJECTS
Heparinized blood samples were obtained from healthy males after an overnight fast. Their ages ranged from 20 to 30 years, none was taking any medications and no history of disorders in carbohydrate metabolism or endocrine system was reported.

ERYTHROCYTE PREPARATION
Approximately 10 ml of fresh heparinised blood was centrifuged for 5 minutes at 1 000 g in a Beckman TJ-6 centrifuge. The plasma together with the white buffy coat layer of the blood cell pellet were aspirated. The red blood cells were resuspended in two parts of normal saline and layered on 3 ml Ficoll-Hypaque. The gradient was centrifuged at 1 500 g for 30 minutes. The supernatant and the upper layer of the cell pellet were aspirated. This isolation procedure was repeated once. All these preceding steps were carried out at room temperature. The cells were washed twice in two parts of buffer G (pH 8) and centrifuged at 4°C at 1 000 g for 10 minutes. Finally the cells were diluted to (3,5 - 5,5) x 10^9 ml^{-1} in cold buffer G (pH 8) and kept on ice until used.

HORMONAL TREATMENT
The cell suspension was incubated at 25°C for 90 minutes in four separate flasks containing insulin, adrenaline, cortisol and no hormone respectively. The final concentration of the hormones was 1,0 $ug \cdot ml^{-1}$ per flask respectively. The suspensions were then centrifuged at room temperature (1 000 g for 10 minutes) after which the cell pellets were washed once in buffer G (pH 6). Incubation was carried out in buffer G(pH 6) at 15°C for 30 minutes to remove or wash off excess hormone on cell surfaces. Finally, the suspensions were washed twice in two parts of cold buffer G (pH 8). Throughout this treatment the cell suspensions were maintained within the range (3,5 - 5,5) x 10^9 ml^{-1}.

INSULIN BINDING ASSAY
The insulin receptor assay is a slightly modified method of Gambhir et al. (32). A set of siliconized glass tubes containing a mixture of 0,2 ng (35 nCi) mono-^{125}I-(Tyr A14) human insulin and different concentrations (0,2; 1,2; 2,2; 5,2; 10,0; 20,0; 50,0; 100,0; 1 000,0; 100 000,0 $ng \cdot ml^{-1}$ final concentration) of unlabelled porcine insulin was prepared. Where necessary, the total volume of the mixture was made up to 100 ul with buffer G(pH8). For each suspension of treated cells, a separate set of tubes was prepared for receptor assay. Into these tubes, 400 ul aliquots of the appropriate red cell suspensions were pipetted. The tubes were incubated at 15°C for 90 minutes with gentle mixing at 20 minute intervals. Following incubation, duplicate 200 ul aliquots of the cell suspensions were pipetted into pre-chilled microfuge tubes containing 200 ul buffer G (pH 8) and 200 ul dibutyl phthalate. The tubes were spun at 5 000 g for 2 minutes in a Beckman Microfuge 12. The buffer layer and

most of the dibutyl phthalate layer were aspirated. Radioactivity in the cell pellets was counted in a Beckman 8 500 gamma counter. Each tube was counted for 5 minutes. The tube with final insulin concentration of 100 000,0 $ng \cdot ml^{-1}$ was used to measure non-specific binding. A duplicate 200 ul aliquot from a pool of leftover cell suspension in the assay tubes was used for total counts.

RESULTS

Hormonal treatment of cells was studied at different temperatures for different periods of time (see table 1). At all temperatures adrenaline caused a decrease in insulin binding with increased preincubation time. This effect levelled off by 90 minutes. However, no significant differences in insulin binding were observed at the different temperatures used for preincubation with adrenaline. Similar results (not shown) were obtained with cortisol. Since temperature appeared to have no significant effect on the pretreatment of cells with adrenaline, they were preincubated at 25°C for 90 minutes throughout the study, prior to measuring insulin binding.

Fig. 1 shows the plot of the competition curve from the control cells and those treated with insulin, adrena-

Table 1 . ADRENALINE EFFECTS ON PERCENTAGE MAXIMUM SPECIFIC INSULIN BINDING

Time (minutes)	% Maximum Specific Insulin Bound		
	15°C	25°C	37°C
0	10,8	10,6	10,6
15	10,1	10,0	7,9
30	9,6	9,1	5,1
45	8,1	8,0	5,0
60	7,5	7,6	4,9
90	6,5	6,2	4,9
120	6,1	6,0	4,5

Red blood cells at a concentration $4,5 \times 10^{9}$ ml^{-1} were incubated in the presence of 1.0 $ug \cdot ml^{-1}$ adrenaline at three different temperatures. At different periods of time, samples were taken from the respective flasks and washed as indicated under Materials and Methods. Insulin binding assay without addition of unlabelled insulin was performed on these cell suspensions for determination of maximum specific binding.

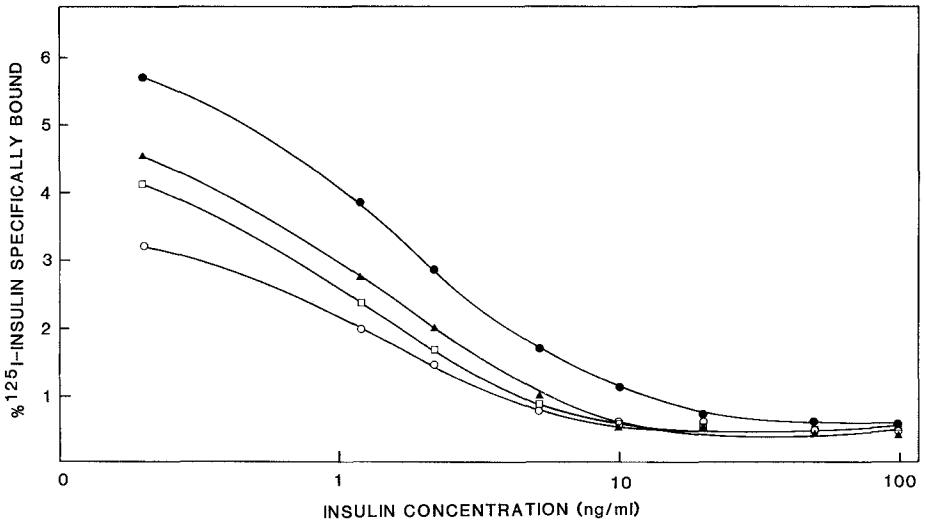

FIG. 1 The effect of hormones on the competition curve of insulin binding to human red blood cells. After pre-incubation of cell suspensions ($4,5 \times 10^9$ cells ml^{-1}) containing no hormone, (●) or 1,0 $ug \cdot ml^{-1}$ (final concentration) of insulin, (○); adrenaline, (□) or cortisol (▲) for 90 minutes at 25°C, insulin binding assay was carried out as described in Materials and Methods. The data was corrected for non-specific binding.

line and cortisol. Treatment of the cells had no effect on the slope of the competition curves, but the curves shifted downwards. This results in the shifting of the 50% maximum insulin displacement to the left. The maximum specific binding (fig. 2) was decreased by 59%, 42% and 27% in cells pretreated with insulin, adrenaline and cortisol respectively.

Treatment of the cells with the hormones resulted in the shifting of the Scatchard plots to the left (fig. 3). This shift was accompanied by a decreased intercept on the Bound axis. In the case of insulin and cortisol, there was no change in the slope of the plots. However, the plot for adrenaline treated cells was characterised by an increased initial slope with no apparent change in the final slope. Fig. 4 shows the analyses of data in fig. 3 using the model for negative co-operativity. Insulin and cortisol had no effect on the upper limit average affinity constant, K_i, whereas an increased K_i was observed in cells treated

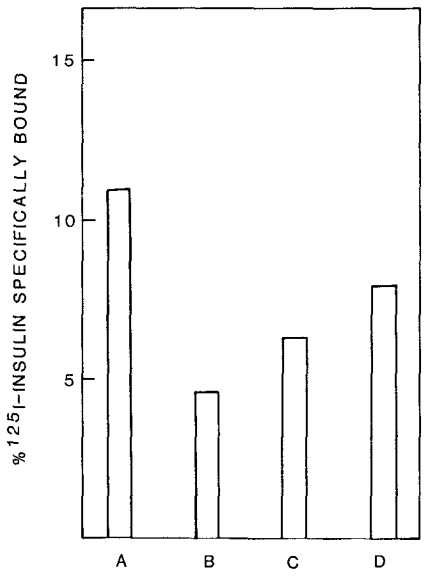

FIG. 2 Percent maximum specific binding of ^{125}I-insulin in cells pre-treated with different hormones. Red blood cells were treated with no hormone (A); insulin, (B); adrenaline, (C) or cortisol, (D) at 25°C for 90 minutes. Then, insulin binding assay was performed without addition of unlabelled insulin.

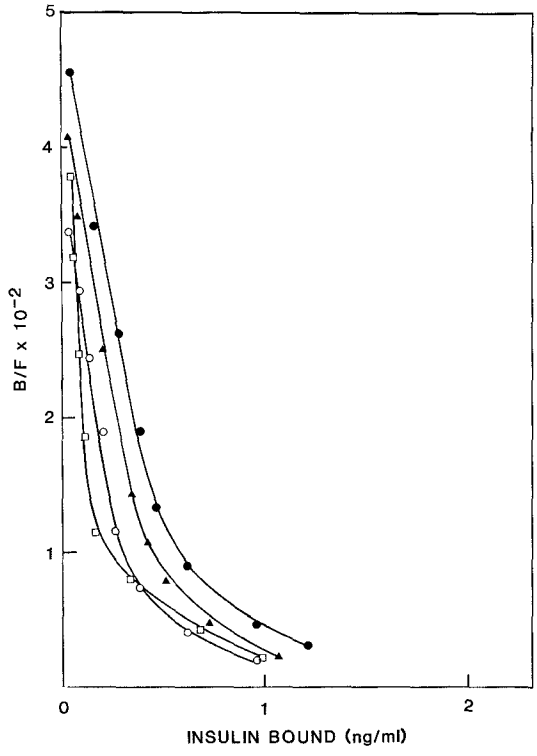

FIG. 3 Scatchard plots of red blood cells preincubated with different hormones. The points in the Scatchard plots were calculated from data in fig. 1. No hormone, (●); insulin, (○); adrenaline, (□) or cortisol (▲).

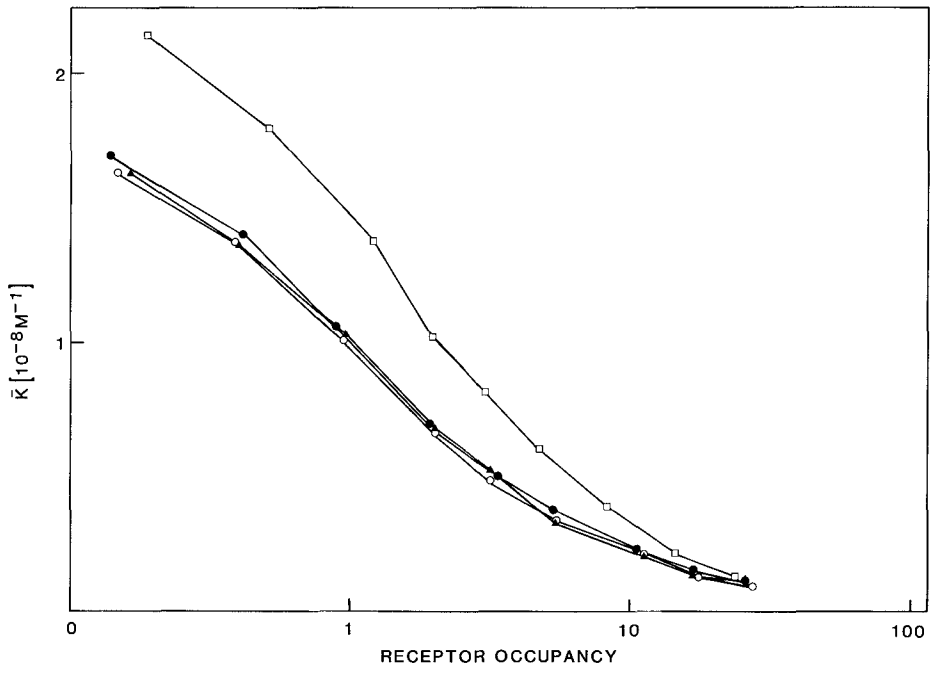

FIG. 4 Average affinity profiles of insulin receptors on red blood cells. Using data from fig. 1 and extrapolations from fig. 3, the model of negative co-operativity was employed to plot average affinity profiles for control cells (●) and cells treated with insulin, (○); adrenaline, (□) or cortisol (▲).

with adrenaline. As the receptors approached binding saturation, hormone treated cells and control cells showed similar lower limit average affinity constants, K_f.

DISCUSSION

The results of this study show that insulin and gluconeogenic hormones have a direct effect on insulin receptors. From the competition curve (fig. 1) and the histograms for maximum specific binding (fig. 2) it is evident that hormonal treatment of red blood cells results in a reduction in insulin binding. In all hormone treated cells the Scatchard plot (fig. 3) showed a reduction in the amount of insulin bound. This indicates that the hormone action causes a decrease in insulin receptor concentration on the cell membrane. In the case of insulin, down-regulation of its receptors has been reported by other investigators (20-22). On the other hand, the results obtained with various steroids on insulin binding are

contradictory (25-30). The findings in this report seem to agree with those of Yasudo and Kitabachi (26).

During stress conditions secretion of adrenaline and cortisol is enhanced whereas that of insulin is suppressed. This results in elevation of blood glucose. This increase in glucose concentration is normally ascribed to stimulation of gluconeogenesis by gluconeogenic hormones. Whilst physiological levels of insulin would tend to maintain normal cell surface receptor number, the occurence of high levels of blood glucose might also be a consequence of down-regulation of insulin receptors by high levels of gluconeogenic hormones. The mechanism by which they exert down-regulation remains to be established. The receptors for insulin and adrenaline are located on the cell membrane, whilst those for cortisol are intracellular. Both these former receptors are coupled to the adenylate cyclase system. In the case of insulin, the adenylate cyclase is inhibited thereby lowering cAMP levels in the cell (33, 34). On the other hand, adrenaline activates the adenylate cyclase thereby causing a rise in cAMP levels (35). In this study we observed that adrenaline, in addition to down-regulation of insulin receptors, caused an increase in the K_i without any significant change in the K_f of the insulin receptors (fig. 4). The mechanism by which the guanine binding proteins (G-protein) modulate the activity of adenylate cyclase is well established (36, 37). In the presence of guanine nucleotides and pertussin sensitive G-protein, the human neutrophil receptors for N-formylpeptide interconvert rapidly between low and high affinity states (37). It is therefore conceivable that a similar mechanism operates when adrenaline increases the K_i of the insulin receptors. This possibility is currently under investigation in our laboratory.

Red blood cells cannot synthesise proteins and therefore the maintenance of receptor concentration on the cell surface is dependent on effective recycling of internalized receptors. It is possible that cortisol, since it manifests its primary effects intracellularly, accomplishes reduction in cell surface receptor concentration by de-

creasing the rate of receptor recycling. In conclusion, our results show that gluconeogenic hormones cause a decrease in insulin receptor number of red blood cells and further investigations should yield valuable information about the mechanism by which they cause down-regulation of the insulin receptors.

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