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# Glucocorticoid Regulation of β-Adrenergic Receptors in 3T3-L1 Preadipocytes

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## SUMMARY

Treatment of 3T3-L1 preadipocytes (fibroblasts) with 250 nM dexamethasone for 48 hr caused a doubling of total β-adrenergic receptors and an increase in $\beta_2$-adrenergic receptor subtype proportion from approximately 50% in controls to 85% in treated cells. The responses to epinephrine and norepinephrine in a whole cell cAMP accumulation assay reflected these changes. The effects of dexamethasone on β-adrenergic receptors were mediated through the glucocorticoid receptor and were time and dose dependent with an $EC_{50}$ of 2.77 ± 0.73 nM for an increase in the proportion of $\beta_2$-adrenergic receptors. The rank order of potency of steroids to effect these changes (betamethasone = dexamethasone > fludrocortisone > hydrocortisone = triamcinolone > aldosterone) correlated with their glucocorticoid potency. [$^3$H]Dexamethasone binding to intact cells yielded a $K_D$ value of 3.47 ± 0.38 nM for binding to the glucocorticoid receptor which correlated well with the $EC_{50}$ for dexamethasone to alter β-adrenergic receptors. Inhibition of [$^3$H]dexamethasone binding by other steroids confirmed that the ability of steroids to regulate β-adrenergic receptors correlated with the affinity of each compound for the 3T3-L1 glucocorticoid receptor. Progesterone, which can bind to the glucocorticoid receptor but has only weak agonist activity, competitively inhibited the ability of dexamethasone to alter β-adrenergic receptors. Protein synthesis, RNA synthesis, and N-linked glycosylation appeared to be necessary for the change in receptor subtype expression and the increase in β-adrenergic receptor number induced by dexamethasone. The present study suggests that regulation of β-adrenergic receptor expression in 3T3-L1 preadipocytes by dexamethasone is a glucocorticoid-specific effect which may require gene activation.

Glucocorticoids have been reported to affect β-adrenergic receptors. Treatment of rats with glucocorticoids has been reported to increase the number of β-adrenergic receptors in the lung (1, 2). In cultured cells, such as $VA_2$, $VA_4$, WI38 (3), ROS 17/2.8 (4), and 3T3-L1 cells (5), dexamethasone, a potent glucocorticoid, has been shown to increase β-adrenergic receptor number. In cultured 3T3-L1 cells, dexamethasone helps to accelerate differentiation from the preadipocyte fibroblast-like state to the adipocyte, increases the number of β-adrenergic receptors identified by radioligand binding, and induces a conversion of expression of the $\beta_1$-adrenergic receptor subtype to the $\beta_2$-adrenergic receptor subtype (5).

β-Adrenergic receptor subtypes can be distinguished by their responses to catecholamine agonists. $\beta_1$-Adrenergic receptors respond with the following rank order: isoproterenol > epinephrine = norepinephrine, whereas $\beta_2$-adrenergic receptors respond to isoproterenol > epinephrine > norepinephrine (6). Antagonists can also distinguish β-adrenergic receptor subtypes. ICI 89.406 is 100-fold selective for $\beta_1$-adrenergic receptors (7) and ICI 118.551 is 100-fold selective for $\beta_2$-adrenergic receptors (8). Functionally, β-adrenergic receptor subtypes respond with different sensitivity to circulating catecholamine hormones and to locally released catecholamine neurotransmitters. $\beta_1$-Adrenergic receptors generally mediate the effects of the locally released neurotransmitter norepinephrine, whereas $\beta_2$-adrenergic receptors respond to the circulating hormone epinephrine. The lungs contain predominantly $\beta_2$-adrenergic receptors which mediate bronchial relaxation. Heart ventricle contains primarily $\beta_1$-adrenergic receptors which regulate heart rate and contractility.

Regulation of receptor subtype and number was studied using the 3T3-L1 cell line. This embryonic mouse fibroblast line was originally cloned by Green and Kehinde (9) for its ability to differentiate into an adipocyte phenotype upon prolonged growth in culture. Subsequent studies showed that the differentiation of these cells is accelerated by, among other things, the presence of high serum (10), insulin (11), dexamethasone, and isobutylmethylxanthine (12). Many studies (13, 14) have characterized changes in enzymes or proteins which occur in these cells upon differentiation. In most cases, preadipocytes were treated with dexamethasone and isobutylmethylxanthine to accelerate the differentiation process and comparisons were

**ABBREVIATIONS:** $^{125}$ICYP, $^{125}$I-iodocyanopindolol; EDTA, ethylenediaminetetraacetic acid; DPBS, Dulbecco's phosphate-buffered saline; TCA, trichloroacetic acid.

made between untreated preadipocytes (fibroblasts) and adipocytes. In one such study, Lai *et al.* (5) observed that β-adrenergic receptors were increased in the adipocytes and that the β-adrenergic receptor subtype which was expressed by the adipocytes was different from that of the preadipocyte. Preadipocytes contained approximately 90% $\beta_1$-adrenergic receptors, whereas adipocytes had 98% $\beta_2$-adrenergic receptors. This alteration in subtype expression was found to be regulated by dexamethasone, since cells differentiated with insulin and isobutylmethylxanthine had β-adrenergic receptors similar to those found in preadipocytes (5). When adipocytes differentiated in the absence of dexamethasone were subsequently treated with dexamethasone, they increased β-adrenergic receptor number and changed subtype from $\beta_1$ to $\beta_2$. Thus, the glucocorticoid effects on β-adrenergic receptors were shown to be independent of differentiation.

We show here that treatment of preadipocytes with dexamethasone causes a change in receptor subtype expression and an increase in receptor number without eliciting any adipose differentiation. This system allows the study of both β-adrenergic receptor number and subtype regulation in a single population of a clonal cell line independent of the differentiation pathway.

The structural distinctions of the two β-adrenergic receptor subtypes are not known, although considerable homologies between them have been observed. Both subtypes can be immunoprecipitated with antibodies to either receptor subtype (15). The mobility of rat fat cell $\beta_1$-adrenergic receptors and S49 mouse lymphoma $\beta_2$-adrenergic receptors in sodium dodecyl sulfate-polyacrylamide gels is identical under reducing or non-reducing conditions (15). The isoelectric points of both β-adrenergic receptor subtypes have been reported to be the same (15). Primary sequence data indicate that species as diverse as hamster ($\beta_2$) and turkey ($\beta_1$) have similar β-adrenergic receptor structure (16). The possibility exists that β-adrenergic receptor subtypes can be interconverted via post-translational modification. The 3T3-L1 system provides a unique opportunity to examine the metabolic requirements for a change of β-adrenergic receptor subtype expression.

Although dexamethasone has been shown to induce an increase in β-adrenergic receptor number and a conversion of β-adrenergic receptor expression from $\beta_1$ to $\beta_2$ in 3T3-L1 cells, relatively little is known about the mechanisms that are involved. The purpose of the present study was to determine if β-adrenergic receptor regulation in 3T3-L1 cells by dexamethasone was mediated through the glucocorticoid receptor and to investigate which metabolic processes may be involved.

## Experimental Procedures

### Materials

3T3-L1 cells were generously donated by Dr. Charles Rubin (Albert Einstein College of Medicine, Bronx, NY). Serum for cell culture was obtained from Hazelton (Denver, PA) and other cell culture reagents were from GIBCO (Grand Island, NY). Steroids, catecholamines, cycloheximide, actinomycin D, and tunicamycin B complex were all obtained from Sigma (St. Louis, MO). [$^3$H]Adenine (12.8 Ci/mmol), [$^{14}$C]cAMP (42.3 mCi/mmol), $^{125}$ICYP (2200 Ci/mmol), [$^3$H]dexamethasone (38.5 Ci/mmol), [$^3$H]leucine (58.4 Ci/mmol), [$^3$H]uridine (40 Ci/mmol), and [$^3$H]glucosamine (42.5 Ci/mmol) were from New England Nuclear (Boston, MA). ICI 118.551 was a gift of Imperial Chemical Industries (England). The tissue solubilizer NCS was obtained from Amersham (Arlington Heights, IL).

### 3T3-L1 Cell Culture

3T3-L1 cells were cultured at 37°, 10% $CO_2$ in complete media consisting of Dulbecco's modification of Eagle's medium with 4.5 g of glucose/liter, supplemented with 10% fetal bovine serum and nonessential amino acids. Immediately upon reaching confluence, cells were treated with the appropriate steroid in complete media. Appropriate controls were treated with complete media alone.

### 3T3-L1 Membrane Preparation

Cell monolayers, grown in 175-cm$^2$ tissue culture dishes, were washed twice with 10 ml of lysis buffer consisting of 5 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4. The monolayers were then incubated for 10 min at 4° with 5 ml of lysis buffer. The cells were scraped from the dishes with a rubber policeman and homogenized by 10 strokes with a Dounce B pestle. The homogenate was centrifuged at 300 × *g* for 10 min and the resulting supernatant was centrifuged at 40,000 × *g* for 15 min. The membrane pellet was resuspended in buffer containing 250 mM sucrose, 75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 1.5 mM EDTA, 0.5 mM dithiothreitol. Membranes were frozen in liquid nitrogen and stored at −70°.

### $^{125}$ICYP Binding Assay

Approximately 100 μg of membranes were incubated for 1 hr at 37° with approximately 50 pM $^{125}$ICYP in the presence or absence of the appropriate concentrations of competing ligand in a total volume of 0.5 ml. To assure that shifts in affinity of ICI 118.551 for $^{125}$ICYP binding were not due to any partial agonist activity of either $^{125}$ICYP or ICI 118.551, GTP ($10^{-4}$ M) was included in the incubation. Bound ligand was separated from free by three 5-ml washes with ice-cold buffer containing 75 mM Tris-HCl, 12.5 mM $MgCl_2$, 1.5 mM EDTA at pH 7.4 using a Brandel cell harvester. Radioactivity associated with the Whatman GF/C filter discs was then counted by a gamma counter. Protein concentrations were determined by the method of Bradford (17) using gamma globulin as standard. Specific binding, which represented 85% of total binding, was determined by subtracting binding in the presence of $10^{-5}$ M alprenolol. This concentration of alprenolol gave results that were comparable to those obtained with $10^{-4}$ M isoproterenol. The proportion of β-adrenergic receptor subtypes was determined by computer modeling of competition binding data obtained with the $\beta_2$-adrenergic receptor-selective antagonist ICI 118.551.

### [$^3$H]cAMP Accumulation Assay

Metabolic labeling and measurement of [$^3$H]cAMP was done by modification of a procedure previously reported (18). The cells were grown to confluence in 12-well plates and then incubated at 37° for 30 min in DPBS containing 1 μCi/ml [$^3$H]adenine. The cells were washed once with 0.5 ml of DPBS and incubated for 30 min at 37° in DPBS, 1 mM isobutylmethylxanthine and the various activators. The reaction was stopped by addition of TCA to 10% and the samples were then centrifuged. The resulting supernatant was run over Dowex and alumina columns to isolate the cAMP-containing fraction as previously described (19). [$^{14}$C]cAMP was used to estimate recovery. The [$^3$H]cAMP was quantified by scintillation counting and the data normalized for cell number.

### [$^3$H]Dexamethasone Binding to Intact Cells

Cells were plated into 28-cm$^2$ dishes and grown to confluence. Cell monolayers were washed twice with warm DPBS and then incubated for 2 hr at 37° with serum-free media containing [$^3$H]dexamethasone and competing ligands. At the end of the incubation period, the cells were scraped from the plates and harvested onto Whatman GF/C filters with a Brandel cell harvester. The cells were washed twice with 5 ml of 37° saline and bound radioactivity was counted in 10 ml of scintillation cocktail. Specific binding, which represented 75% of total binding, was determined by subtracting binding in the presence of a 1000-fold excess of unlabeled hydrocortisone.

### Inhibition of Metabolic Processes

Cells were plated into 175-cm² dishes and six-well plates and allowed to grow to confluence. At that time cells were treated in complete media with or without dexamethasone or the appropriate inhibitor and further incubated for 24 hr. The six-well plates were used to determine the per cent inhibition of radioactive isotope incorporation. Experiments were performed with a range of concentrations (0.05–5 μg/ml) of inhibitors to determine the optimal concentrations for these studies. In the experiments reported, no significant effects on cell viability were observed.

**Protein synthesis.** Cells were treated in complete media for 24 hr with or without dexamethasone and with or without 1 μg/ml cycloheximide to inhibit protein synthesis. At the same time, cells in six-well plates were also incubated with 10 μCi/ml [³H]leucine or [³H]uridine to determine the extent that cycloheximide inhibited [³H]leucine incorporation into protein and, as control, [³H]uridine incorporation into RNA. At the end of the incubation period, cells in 175-cm² dishes were harvested and β-adrenergic receptor subtype and number were determined by radioligand binding. For the six-well plates, one well from each treatment was trypsinized and counted. The media from the wells containing [³H]leucine or [³H]uridine were removed, 1.0 ml of 10% TCA was added, the wells were scraped, and the resulting suspension was centrifuged in a Microfuge for 2 min. The pellet was washed once with 10% TCA and then solubilized with NCS. The radioactivity in the pellet was counted by a Beckman liquid scintillation counter and the cpm were normalized for cell number. Per cent inhibition was calculated by comparing radioactivity incorporated in the presence or absence of inhibitor.

**RNA synthesis.** This procedure was the same as for cycloheximide, except that 1 μg/ml actinomycin D was used to inhibit RNA synthesis. [³H]Uridine (10 μCi/ml) was used to detect inhibition of RNA synthesis and [³H]leucine (10 μCi/ml) was used to determine the extent of protein synthesis inhibition by actinomycin D.

**N-linked glycosylation.** Cells were treated the same as with cycloheximide, except 0.5 μg/ml tunicamycin was used to inhibit N-linked glycosylation and 10 μCi/ml [³H]glucosamine was added to six-well plates to assess sugar incorporation. A 6-hr preincubation with tunicamycin preceded addition of labeled sugar and dexamethasone to allow tunicamycin to take effect (20). After a 24-hr incubation with tunicamycin, cells in 175-cm² dishes were harvested and membranes were prepared and stored for radioligand binding. To specifically assess inhibition of N-linked glycosylation in the six-well plates, the media were removed and O-linked sugars were hydrolyzed by incubation in 1 ml of 0.1 N NaOH for 18 hr at 37° as previously described (20). One hundred μl of 100% TCA were added and the mixture was centrifuged in a Microfuge. The pellet was washed with 1 ml of 10% TCA and then solubilized with solubilizing reagent NCS.

The amount of [³H]leucine, [³H]uridine, and [³H]glucosamine incorporated by controls or treated cells was not affected by the presence of dexamethasone. Cycloheximide inhibited protein synthesis by 84% and RNA synthesis by 47%. Actinomycin D inhibited RNA synthesis by 96% and protein synthesis by 59%. Tunicamycin inhibited glycosylation by 64% and protein synthesis by 32%.

### Data Analysis

Data generated by competition and saturation binding experiments were analyzed by a non linear least squares curve fit performed by the computer program SCATFIT (21). The error of the duplicate data points from the binding assays was < 10%. The error of the binding parameters determined by computer fitting of the competition curves to determine receptor subtype was < 8%. The Student's *t* test was used to calculate the statistical difference between paired sets of data.

## Results

Dexamethasone treatment increased the proportion of $\beta_2$-adrenergic receptors in 3T3-L1 fibroblasts (Fig. 1). ICI 118.551

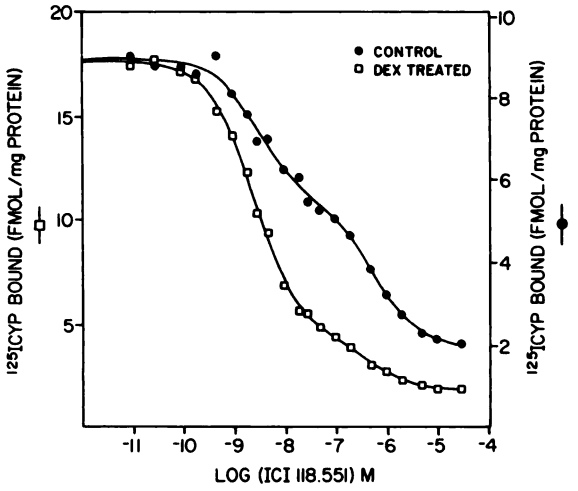


**Fig. 1.** Competition of $^{125}$ICYP binding by the $\beta_2$-adrenergic receptor subtype-selective antagonist ICI 118.551 to membranes prepared from control and dexamethasone-treated 3T3-L1 cells. 3T3-L1 cells were treated for 48 hr with (□) or without (●) 250 nM dexamethasone (*DEX*), and a partially purified membrane fraction was prepared from the cells (see Experimental Procedures). β-Adrenergic receptors were then assayed with the radioligand $^{125}$ICYP (see Experimental Procedures) in the presence of varying concentrations of ICI 118.551. The curves through the data represent the best fit of a computer-assisted analysis of the data which calculated the percentage of β-adrenergic receptor subtypes at 45% $\beta_2$ in control and 85% $\beta_2$ in dexamethasone-treated cells. The data represent one experiment assayed in duplicate. The experiment was repeated 39 times with similar results.

TABLE 1

**$K_D$ and $B_{max}$ values for $^{125}$ICYP binding to membranes from control and dexamethasone-treated 3T3-L1 fibroblasts**

3T3-L1 cells were treated with or without 250 nM dexamethasone for 48 hr. Binding to membranes was assayed in the presence of varying concentrations of $^{125}$ICYP ranging from 1 to 200 pM. Alprenolol ($10^{-6}$ M) was used to determine nonspecific binding. Data were analyzed by the computer program SCATFIT. The values represent the means ± standard errors of three experiments assayed in duplicate.

| | $K_D$ values | $B_{max}$ values |
|---|---|---|
| | *pM* | *fmol/mg of protein* |
| Control | 25.3 ± 3.1 | 28.2 ± 3.9 |
| Dexamethasone treated | 22.8 ± 3.5 | 47.7 ± 5.4 |

has 100-fold greater affinity for $\beta_2$-adrenergic receptors than for $\beta_1$-adrenergic receptors (8) and was therefore employed in competition binding assays with the nonselective radiolabeled antagonist $^{125}$ICYP to determine the proportions of β-adrenergic receptor subtypes. Because of the high selectivity of ICI 118.551, computer modeling of the data can detect small (<20%) differences in the proportion of receptor subtypes (22). Untreated 3T3-L1 cells displayed 55% and 45% $\beta_1$-adrenergic receptors and $\beta_2$-adrenergic receptors, respectively (Fig. 1). After treatment with 250 nM dexamethasone for 48 hr, 85% of the β-adrenergic receptors were of the $\beta_2$-adrenergic receptor subtype. Dexamethasone-treated cells bound approximately 18 fmol of $^{125}$ICYP/mg of protein, whereas the control cells bound only 9 fmol of $^{125}$ICYP/mg of protein (Fig. 1). This increase in $^{125}$ICYP binding induced by dexamethasone treatment suggested an increase in β-adrenergic receptor number and was further characterized by saturation binding analysis.

The increase in total β-adrenergic receptors was not due to a change in receptor affinity for the radioligand $^{125}$ICYP. Analysis of saturation binding data (Table 1) revealed that dexamethasone treatment did not alter the $K_D$ value for $^{125}$ICYP

binding but did increase the $B_{max}$ for binding by approximately 2-fold.

The increase in the percentage of $\beta_2$-adrenergic receptors was due both to an increase in $\beta_2$-adrenergic receptor expression and to a decrease in $\beta_1$-adrenergic receptor expression (Fig. 2). Total specific $^{125}$ICYP bound and the proportions of $\beta$-adrenergic receptor subtypes were determined as a function of time of treatment with dexamethasone. These data were combined in Fig. 2 to show the change in fmol of $^{125}$ICYP bound to each receptor subtype compared to time-matched controls. No effect of dexamethasone was observed at 6 hr. A slight change in receptor subtype proportions was seen before 24 hr. The decrease in $\beta_1$-adrenergic receptors at 14 hr was not significantly different from the decrease observed at longer time points. The increase in $\beta_2$-adrenergic receptors was maximal at approximately 72 hr.

Whole cell cAMP accumulation data (Fig. 3) reflected a functional change in receptor subtype and number effected by dexamethasone. In control preadipocytes, epinephrine and norepinephrine induced cAMP accumulation with similar potency, suggesting a $\beta_1$-adrenergic receptor phenotype, whereas in dexamethasone-treated preadipocytes, epinephrine was more potent in stimulating cAMP accumulation than norepinephrine, suggesting a $\beta_2$-adrenergic receptor phenotype. The increase in the total amount of [$^3$H]cAMP generated by epinephrine and norepinephrine in dexamethasone-treated cells ($4.0 \times 10^3$ cpm [$^3$H]cAMP/$10^5$ cells compared to $2.5 \times 10^3$ cpm [$^3$H]cAMP/$10^5$ cells in controls) reflected the increase in total $\beta$-adrenergic receptors which was observed upon dexamethasone treatment. cAMP accumulation elicited by 10 $\mu$M forskolin did not change with dexamethasone treatment (Fig. 2), suggesting that the changes in agonist-induced cAMP accumulation observed were receptor mediated.

If the observed receptor regulation was mediated through the glucocorticoid receptor, then the ability of the compounds to regulate $\beta$-adrenergic receptors should correlate with their glucocorticoid potency. Dexamethasone, a potent glucocorticoid compound, affected $\beta$-adrenergic receptors at concentrations as low as 1 nM (Fig. 4) and had maximal effects at concentrations between 100 nM and 1 $\mu$M. The effects of dexamethasone on $\beta$-adrenergic receptor number were comparable to the effects seen on receptor subtype, although slightly higher concentrations of dexamethasone were required to see the effects on receptor number. To determine more conclusively if glucocorticoid activity induced these effects, a series of glucocorticoids was studied. Cells were treated with increasing concentrations (1–1000 nM) of selected compounds. The $EC_{50}$ values for altering receptor subtype (compared with the effect elicited by 250 nM dexamethasone) are shown in Table 2. As a measure of glucocorticoid activity, the relative anti-inflammatory potency (23) for each compound is presented for comparison. The ability of each agent to regulate $\beta$-adrenergic receptor subtype (betamethasone = dexamethasone > fludrocortisone > hydrocortisone = triamcinolone > aldosterone) correlated well with its glucocorticoid potency. Dexamethasone and betamethasone, the most potent glucocorticoids tested, had the lowest $EC_{50}$ values. Aldosterone, which has predominantly mineralocorticoid activity but also has slight glucocorticoid activity, affected $\beta$-adrenergic receptors, but only at concentrations greater than 1 $\mu$M. To assure that aldosterone's effects were not mediated through the mineralocorticoid receptor but were instead caused by its binding to the glucocorticoid receptor, spironolactone (a high affinity mineralocorticoid antagonist) was used to block competitively aldosterone's mineralocorticoid-mediated effects. Aldosterone's ability to regulate $\beta$-adrenergic receptors at 1 $\mu$M was not blocked by the addition of 1 $\mu$M spironolactone (data not shown). Except for progesterone, compounds with no glucocorticoid potency had no effect on $\beta$-adrenergic receptors (Table 2). Progesterone had a slight effect (40% of 10 nM dexamethasone's effects at 10 $\mu$M progesterone) on $\beta$-adrenergic receptor subtype regulation and was considered to be a partial agonist (see Discussion).

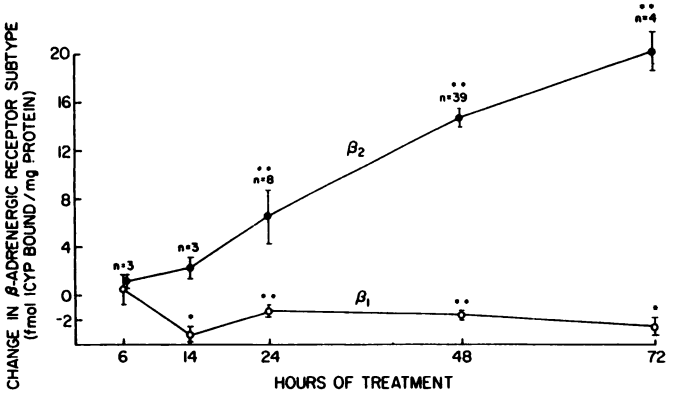


**Fig. 2.** Time course of $\beta$-adrenergic receptor subtypes changes observed upon dexamethasone treatment of 3T3-L1 cells. Changes compared to time-matched controls in fmol of $^{125}$ICYP bound to either $\beta_1$-adrenergic receptors (○) or $\beta_2$-adrenergic receptors (●) were determined after varying times of treatment with 250 nM dexamethasone. The fmol of $\beta$-adrenergic receptor subtypes were calculated using total $^{125}$ICYP binding minus nonspecific binding (in the presence of $10^{-5}$ M alprenolol) to determine specific binding, and computer analysis of data from competition binding experiments with the $\beta_2$-adrenergic receptor-selective antagonist ICI 118.551 to determine the proportions of $\beta$-adrenergic receptor subtypes (see Fig. 1). The data represent the means ± standard errors. *, the difference from time-matched controls is significantly different, $p < 0.025$. **, the difference from time-matched controls is significantly different, $p < 0.01$.

Since we believed that dexamethasone induced these effects via a specific, high affinity glucocorticoid receptor, these receptors were characterized in 3T3-L1 fibroblasts by [$^3$H]dexamethasone binding to intact monolayers. Computer analysis of data from saturation analysis yielded a $K_D$ of 3.47 ± 0.38 nM and a $B_{max}$ of 50,100 ± 2,200 sites/cell ($n = 3$). The $K_D$ value for [$^3$H]dexamethasone binding correlated very well with the 2.77 nM $EC_{50}$ value for dexamethasone regulation of $\beta$-adrenergic receptor subtype. Competition binding (Fig. 5) showed that the ability of compounds to compete for [$^3$H]dexamethasone binding correlated with their glucocorticoid potency and with their ability to regulate $\beta$-adrenergic receptors (Table 2). $K_D$ values for compounds to bind the glucocorticoid receptor were determined by computer modeling of data from competition binding experiments with [$^3$H]dexamethasone (Fig. 5) and are as follows: dexamethasone, 4.8 ± 0.7 nM; hydrocortisone, 24 ± 6.2 nM; aldosterone, 116 ± 21 nM; progesterone, 179 ± 44 nM. These $K_D$ values correlated well with the $EC_{50}$ values for altering $\beta$-adrenergic receptors.

At high concentrations progesterone can bind to the glucocorticoid receptor to prevent binding of other ligands (24) and could therefore be used to block the glucocorticoid receptor-mediated effects of dexamethasone (25, 26). Since progesterone in our system is recognized by the glucocorticoid receptor (Fig. 5) and had only slight effects on $\beta$-adrenergic receptors at

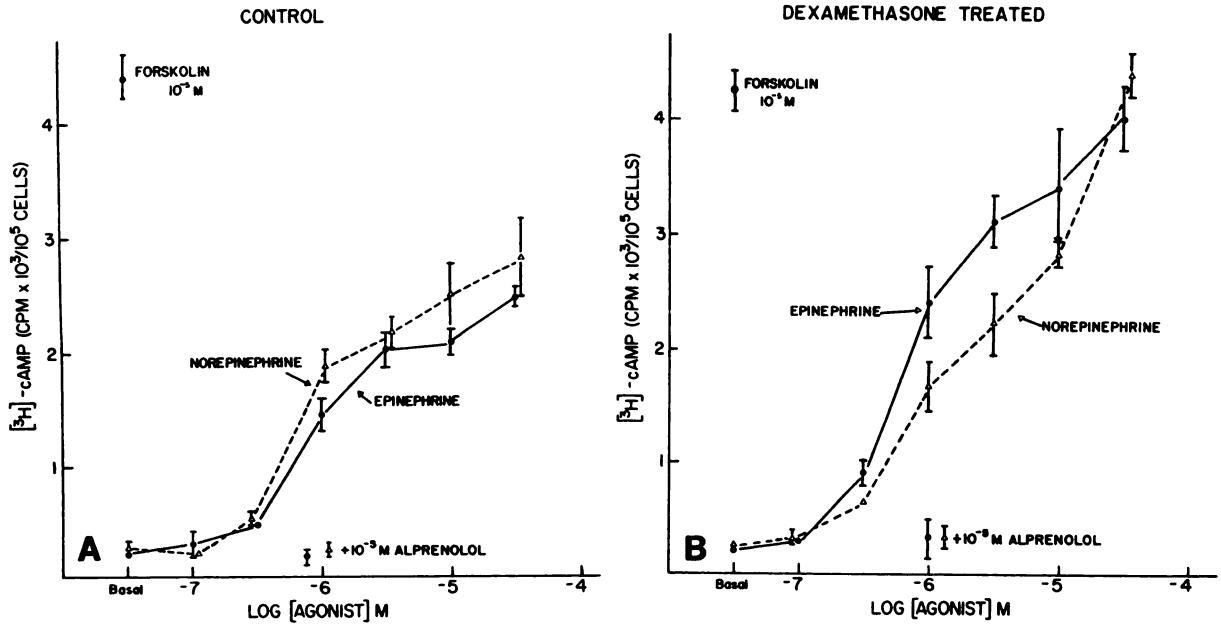


**Fig. 3.** Agonist-induced cAMP accumulation in 3T3-L1 cells treated for 48 hr with or without 250 nM dexamethasone. 3T3-L1 cells were treated with either media alone (control) (A) or with 250 nM dexamethasone for 48 hr (B). Agonist-induced cAMP accumulation was measured as described in Experimental Procedures. [$^3$H]cAMP accumulation was measured in response to epinephrine (●) and norepinephrine (△). Alprenolol ($10^{-5}$ M) was added to $10^{-6}$ M concentrations of each agonist to determine nonspecific cAMP accumulation. Forskolin ($10^{-5}$ M) was assayed alone and is shown above the basal point on each graph. The data represent the means ± standard errors of triplicate determinations. The experiment was repeated once with similar results.

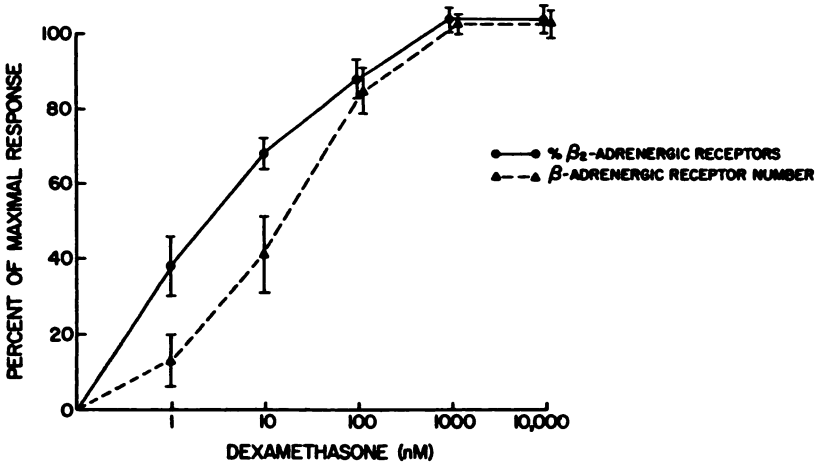


**Fig. 4.** Dose response for dexamethasone's effects of increasing $\beta_2$-adrenergic receptor proportions and increasing β-adrenergic receptor number. 3T3-L1 cells were treated for 48 hr with increasing concentrations of dexamethasone. Membranes were prepared and incubated with 50 pM $^{125}$ICYP in the presence or absence of $10^{-5}$ M alprenolol to determine specific binding. The same membranes were assayed with $^{125}$ICYP and varying concentrations of ICI 118.551 to determine the proportions of β-adrenergic receptor subtypes. The change in receptor number (▲) or subtype (●) induced by each dose of dexamethasone was compared with the response elicited by a maximal dose (250 nM) of dexamethasone. The percentage of maximal response for each dose is shown. The data represent the means ± standard errors of six experiments performed in duplicate.

concentrations as high as 10 μM (Table 2), it was used to inhibit the effects of dexamethasone. At concentrations which blocked [$^3$H]dexamethasone binding, progesterone significantly inhibited the effects of a submaximal dose (10 nM) of dexamethasone on β-adrenergic receptors (Table 3). Receptor number in cells treated simultaneously with dexamethasone and progesterone was not significantly different from that of controls, with $p < 0.25$. A submaximal dose of dexamethasone was selected since a larger dose of a high affinity ligand such as dexamethasone would prevent inhibition by a lower affinity competitor such as progesterone. The small proportional increase in β-adrenergic receptors over control induced by combined dexamethasone and progesterone treatment was similar in magnitude to the change induced by 10 μM progesterone alone (65% ± 3.9% for 10 μM progesterone compared to 57% ± 2.8% for control, $n = 3$).

To begin to elucidate the mechanism by which dexamethasone alters β-adrenergic receptor expression, we studied the effects of metabolic inhibitors on β-adrenergic receptor regulation by dexamethasone. In all of these experiments the cells were viable as determined by trypan blue exclusion. Experiments with metabolic inhibitors could not be carried out for longer than 24 hr since the drugs were toxic at longer treatment times. Treatment of 3T3-L1 cells with cycloheximide, actinomycin D, or tunicamycin prevented the dexamethasone-induced increase in β-adrenergic receptor number (Table 4A) and the change in subtype expression (Table 4B). Inhibition of protein synthesis by 84% using 1 μg/ml cycloheximide completely inhibited the change in subtype expression (Table 4B) and the increase in receptor number (Table 4A) caused by dexamethasone. Actinomycin D at 1 μg/ml inhibited RNA synthesis by 96% and concomitantly inhibited dexamethasone's effects on β-adrenergic receptors (Table 4). The β-adrenergic receptor protein contains sites for glycosylation (27, 28). To

TABLE 2

**$EC_{50}$ values for compounds to increase $\beta_2$-adrenergic receptor subtype proportion**

3T3-L1 cells were incubated for 48 hr with increasing concentrations of the indicated compounds. Membranes were assayed with $^{125}$ICYP and varying doses of ICI 118.551 to determine receptor subtype. The effect of these compounds on $\beta$-adrenergic receptor subtype was compared with that of 250 nM dexamethasone which was defined as 100%. The $EC_{50}$ value (± standard error) represents the concentration of compound which altered receptor subtype 50% (compared to 100% by 250 nM dexamethasone). The relative anti-inflammatory potency (23) of each compound is also presented for comparison.

| Compound | $EC_{50}$ | Relative Anti-inflammatory potency |
|---|---|---|
| | *nM* | |
| Betamethasone | 2.77 ± 0.73 ($n$ = 3) | 25 |
| Dexamethasone | 2.93 ± 0.90 ($n$ = 6) | 25 |
| Fludrocortisone | 6.00 ± 3.06 ($n$ = 3) | 10 |
| Hydrocortisone | 28.25 ± 10.48 ($n$ = 4) | 1 |
| Triamcinolone | 24.25 ± 12.03 ($n$ = 4) | 5 |
| Aldosterone | 187 ± 51 ($n$ = 4) | 0.3 |
| Progesterone | Slight effect at 10 μM ($n$ = 3) | 0 |
| Thyroxine | No effect at 1 μM ($n$ = 2) | 0 |
| Estradiol | No effect at 1 μM ($n$ = 3) | 0 |
| Dihydrotestosterone | No effect at 10 μM ($n$ = 2) | 0 |

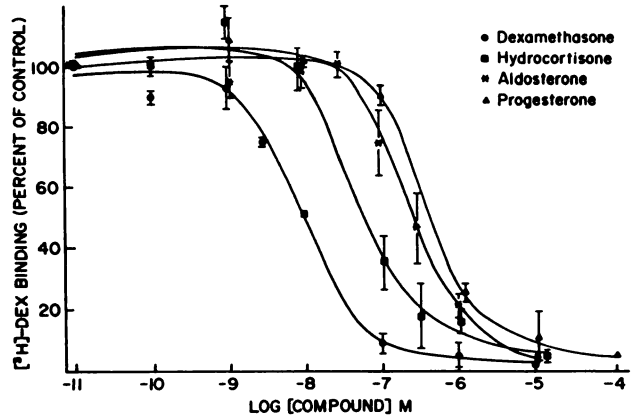


**Fig. 5.** Competition of [$^3$H]dexamethasone binding by selected steroids. Cell monolayers were incubated with 5 nM [$^3$H]dexamethasone (*[$^3$H]-DEX*) and varying concentrations of the indicated steroids as described in Experimental Procedures. The data points represent the means ± standard errors of three experiments assayed in duplicate. Data from each of these experiments were computer-modeled to determine the $K_D$ values for these compounds. The calculated values are as follows: dexamethasone, 4.85 ± 0.70 nM, hydrocortisone, 24 ± 6.2 nM; aldosterone, 116 ± 21 nM; progesterone, 179 ± 44 nM.

TABLE 3

**Progesterone inhibition of dexamethasone-induced $\beta$-adrenergic receptor regulation**

Cells were treated for 48 hr with 10 nM dexamethasone with or without 10 μM progesterone. The cells were harvested and membranes assayed for $\beta$-adrenergic receptor subtype by radioligand binding. The data represent the means ± standard errors of three paired experiments.

| Treatment | Percentage of $\beta_2$-adrenergic receptors |
|---|---|
| Control | 57 ± 2.8 |
| Dexamethasone | 77 ± 3.1 |
| Dexamethasone + progesterone | 65 ± 3.9[a] |

[a] The presence of progesterone significantly inhibited the response of dexamethasone, $p < 0.025$.

TABLE 4

**Effect of protein synthesis, RNA synthesis, and glycosylation inhibitors on $\beta$-adrenergic receptor regulation in 3T3-L1 cells by dexamethasone**

Partially purified membranes were prepared from control and dexamethasone (250 nM)-treated 3T3-L1 cells (see Experimental Procedures) which were simultaneously treated with or without either 1 μg/ml cycloheximide to inhibit protein synthesis, 1 μg/ml actinomycin D mannitol to inhibit RNA synthesis, or 0.5 μg/ml tunicamycin B complex to inhibit N-linked glycosylation. Cycloheximide inhibited protein synthesis by 84%, actinomycin D inhibited RNA synthesis by 96%, and tunicamycin inhibited glycosylation by 64% (see Experimental Procedures). In part A, membranes were incubated with 50 pM $^{125}$ICYP (see Experimental Procedures) in the presence or absence of $10^{-5}$ M alprenolol to determine specific binding. In part B, computer modeling of data generated from competition by ICI 118.551 for $^{125}$ICYP binding allowed determination of the proportions of $\beta$-adrenergic receptor subtypes. The data represent the means ± standard errors of three experiments.

| Treatment | | A. $\beta$-Adrenergic receptor number | |
|---|---|---|---|
| | | Control | Dexamethasone treated |
| | | *fmol/mg of protein* | |
| Cycloheximide | none | 10 ± 1.1 | 16 ± 2.3 |
| | 1 μg/ml | 9.7 ± 0.3[a] | 9.0 ± 0.6[b] |
| Actinomycin D | none | 11 ± 1.2 | 17 ± 4.6 |
| | 1 μg/ml | 7.8 ± 2.3[a] | 7.3 ± 1.4[b] |
| Tunicamycin | none | 21 ± 4.5 | 32 ± 6.6 |
| | 0.5 μg/ml | 13 ± 4.0[c] | 15 ± 4.1[c] |

| Treatment | | B. Percentage of $\beta_2$-adrenergic receptors | |
|---|---|---|---|
| | | Control | Dexamethasone treated |
| Cycloheximide | none | 55 ± 2.5 | 78 ± 0.9 |
| | 1 μg/ml | 54 ± 1.1[a] | 57 ± 2.9[c] |
| Actinomycin D | none | 42 ± 7.4 | 69 ± 7.6 |
| | 1 μg/ml | 48 ± 6.4[b] | 50 ± 8.3[c] |
| Tunicamycin | none | 54 ± 1.8 | 79 ± 0.7 |
| | 0.5 μg/ml | 50 ± 0.7[a] | 58 ± 0.9[c] |

[a] The addition of inhibitor did not cause a significant difference, $p < 0.10$.
[b] The addition of inhibitor caused a statistically significant difference, $p < 0.05$.
[c] The addition of inhibitor caused a statistically significant difference, $p < 0.025$.

examine whether the change in subtype and increase in receptor number observed upon dexamethasone treatment required N-linked glycosylation, we used tunicamycin at 0.5 μg/ml to inhibit this process. N-linked glycosylation was inhibited 64%. The decrease in $\beta$-adrenergic receptor number seen upon tunicamycin treatment was not due to normal protein turnover since receptor levels did not decrease with actinomycin D or cycloheximide treatment. The reason for this decrease cannot be explained at this time. All effects on $\beta$-adrenergic receptors by dexamethasone were blocked by this dose of tunicamycin.

## Discussion

Dexamethasone induced a dose- and time-dependent increase in $\beta_2$-adrenergic receptor expression, a decrease in $\beta_1$-adrenergic receptor expression, and an increase in total $\beta$-adrenergic receptor number. The receptor increase was not attributable to a change in receptor affinity for the radioligand $^{125}$ICYP, but was instead due to an increase in $\beta$-adrenergic receptor number. Dexamethasone's effects were observed before 24 hr and at concentrations of dexamethasone as low as 1 nM. $\beta_1$-Adrenergic receptor levels decreased at 14 hr and remained decreased compared to time-matched controls.

The increase in $\beta_2$-adrenergic receptors seen upon dexamethasone treatment (Fig. 2) was probably due to an increase in $\beta$-adrenergic receptor synthesis or synthesis of a protein that could modify existing unfunctional $\beta_2$-adrenergic receptors. The $\beta_2$-adrenergic receptor increase was probably not solely due to a conversion of preexisting $\beta_1$-adrenergic receptors to $\beta_2$-adrenergic receptors since an increase in total $\beta$-adrenergic recep-

tors was observed. The relatively long time course and low levels of dexamethasone required suggested that dexamethasone activates gene transcription through the glucocorticoid receptor to mediate these effects. Dexamethasone's effects on β-adrenergic receptors are subtype specific since $\beta_2$-adrenergic receptors increase whereas $\beta_1$-adrenergic receptors decrease. The small, but significant decrease in $\beta_1$-adrenergic receptors seen after dexamethasone treatment probably does not reflect normal protein turnover since inhibiting protein turnover with cycloheximide at levels which inhibit protein synthesis 90% did not cause a decrease in β-adrenergic receptor levels until after 24 hr (Table 4A). The possibility cannot be ruled out that cycloheximide indirectly reduces the rate of protein degradation to cause the observed effect.

Whole cell cAMP accumulation stimulated by epinephrine and norepinephrine (Fig. 3) showed that β-adrenergic receptors in control and dexamethasone-treated preadipocytes have the functional characteristics of $\beta_1$-adrenergic receptors and $\beta_2$-adrenergic receptors, respectively. This assay was performed on intact cells because we and others (29) have found that membranes made from 3T3-L1 preadipocytes do not respond to catecholamine agonists. Whole cells have a functionally coupled β-adrenergic receptor-adenylate cyclase system when assayed by either metabolic labeling (Fig. 3) or by radioimmunoassay[1] for cAMP. The increase in the number of β-adrenergic receptors indicated by saturation binding of $^{125}$ICYP was reflected in the increase in the maximal amount of cAMP accumulated in control and dexamethasone-treated cells.

The correlation between glucocorticoid-dependent activity and the ability of various compounds to alter β-adrenergic receptors provides additional data supporting the concept that β-adrenergic receptors are regulated by glucocorticoids in 3T3-L1 preadipocytes. To further support this hypothesis, we characterized the glucocorticoid-binding sites in these cells (Fig. 5). The data suggest that preadipocyte β-adrenergic receptors are regulated via the glucocorticoid receptor and that the mechanism of this regulation is analogous to other types of glucocorticoid-induced processes (30). The $K_D$ for binding to these receptors correlated with the potency of effects on β-adrenergic receptors (Table 2) which provides direct evidence for the hypothesis that these effects are due to glucocorticoid activity.

Since progesterone has been shown to be an antagonist at the glucocorticoid receptor in some systems (25, 26), it was employed to block dexamethasone's regulation of β-adrenergic receptors. The ability of progesterone to inhibit [$^3$H]dexamethasone binding (Fig. 5) indicated that it did bind to the glucocorticoid receptor and, since there were only slight effects on β-adrenergic receptors at concentrations as high as 10 μM (Table 2), progesterone was not acting as a full agonist in our cells. Progesterone occupied the glucocorticoid receptor and inhibited the ability of dexamethasone to regulate β-adrenergic receptors. This provided further evidence that dexamethasone's effects were glucocorticoid receptor mediated.

Several apparent discrepancies exist between these data on β-adrenergic receptor regulation in 3T3-L1 cells by dexamethasone and data previously reported (5). In our study, the proportion of $\beta_2$-adrenergic receptors in control preadipocytes was higher (50% versus 10%) and the β-adrenergic receptors per cell in control and dexamethasone-treated preadipocytes were lower. These discrepancies could be due to the fact that our studies were done in the preadipocyte form of 3T3-L1 cells whereas the other studies (5) compared preadipocyte and adipocyte β-adrenergic receptors. Other possible reasons for differences in data obtained from these cells include differences in cell culture conditions such as serum, levels of confluence upon treatment, or time of passage. Most notable is that the same qualitative effects of increasing $\beta_2$-adrenergic receptor and decreasing $\beta_1$-adrenergic receptor proportions, and increasing total β-adrenergic receptor number, were observed here as in previous work (5).

We have chosen to study β-adrenergic receptor regulation by dexamethasone in the preadipocyte form of the 3T3-L1 cell line since this is a simplified system which allows the study of β-adrenergic receptor regulation in only one form of 3T3-L1 cells. In this system, the changes in cell metabolism which occur upon differentiation and a comparison of receptors in two separate populations of a cell clone are avoided. The 3T3-L1 cell line is assumed to be clonal although the possibility does exist that our cells consist of subclones which express one receptor subtype or another. There is precedent, though, for the two β-adrenergic receptor subtypes to be expressed in a clonal cell line (31). Regardless, in 3T3-L1 cells, dexamethasone regulates both receptor subtypes independently since the levels of one subtype increase while levels of the other decrease.

Protein synthesis, RNA synthesis, and glycosylation appear to be required for the regulation of β-adrenergic receptor subtype expression. Previous studies (20, 32) have examined the effects of metabolic inhibitors on the recovery of receptors from a down-regulated state, but none have addressed the metabolic requirements for the specific induction of β-adrenergic receptors. Definitive conclusions on dexamethasone's induction of new β-adrenergic receptor proteins or on the role of glycosylation in receptor processing cannot be drawn from the current experiments since the metabolic inhibitors used may have adverse effects on other cell functions. There is precedent for use of these compounds under the experimental conditions employed in the present studies to investigate the role of metabolic processes in receptor regulation (20, 32, 33). Our data are consistent with the notion that dexamethasone acts through the glucocorticoid receptor to cause gene activation resulting in β-adrenergic receptor regulation. Previous studies suggest that there are considerable similarities in β-adrenergic receptor subtype structures (15). Post-translational modification may define receptor subtype. Tunicamycin inhibition of β-adrenergic receptor induction suggests that the β-adrenergic receptors must be glycosylated to become functionally incorporated into the membrane. These data support previous reports (20). Our results allow the possibility that receptor subtypes may be converted by glycosylation.

The physiological relevance of glucocorticoid regulation of β-adrenergic receptor number may be apparent in the body's response to stress. Both catecholamines and glucocorticoids are released in response to stress. Catecholamines bind to β-adrenergic receptors to increase adrenergic activity within minutes of their release. Stimulation by catecholamines for an extended time will cause down-regulation of β-adrenergic receptors, resulting in decreased adrenergic activity. Glucocorticoids may compensate for the down-regulation by increasing receptor

[1] J. S. Stadel, K. S. Poksay, M. T. Nakada, and S. T. Crooke, manuscript in preparation.

expression. In response to stress, the adrenals secrete predominantly the catecholamine hormone epinephrine which binds with high affinity to $\beta_2$-adrenergic receptors. Glucocorticoids increase the number of functional $\beta_2$-adrenergic receptors. The decrease in $\beta_1$-adrenergic receptors is presumably not due simply to receptor turnover, as the decrease in $\beta_1$-adrenergic receptors is more rapid with dexamethasone than in its absence.

The cloning of the $\beta$-adrenergic receptor gene (34) will allow the study of the genetics of $\beta$-adrenergic receptor regulation. The characterization of 3T3-L1 $\beta$-adrenergic receptor regulation by dexamethasone establishes these cells as a system which allows study not only of receptor number regulation but also of receptor subtype regulation at a genetic level. Since the differences between two $\beta$-adrenergic receptor subtypes are not known and new tools are now available for their study, this system may prove invaluable for the elucidation of receptor subtype structure and genetics.

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