Glucocorticoid Modulation of β-Adrenergic Receptors of Cultured Rat Arterial Smooth Muscle Cells

ALLAHYAR JAZAYERI AND WALTER J. MEYER, III

SUMMARY Since both glucocorticoids and catecholamines are involved in the regulation of normal blood pressure, we investigated the modulation of β-adrenergic receptors of cultured rat arterial smooth muscle cells by glucocorticoids. The synthetic glucocorticoids dexamethasone and RU 28362, at 10^{-8} M concentration, increased maximum β-adrenergic binding but had no effect on the dissociation constant (K_d). Each steroid caused an increase in maximum [3H]dihydroalprenolol binding over the concentration range of 10^{-8} to 10^{-4} M, but not at 10^{-9} M. The glucocorticoid effect on β-adrenergic receptors of arterial smooth muscle cells required a minimum of 20 hours of incubation in the presence of the steroid and was significantly inhibited by cycloheximide (10 μg/ml), indicating that the glucocorticoid effect required protein synthesis. The effect of dexamethasone on [3H]dihydroalprenolol binding was significantly inhibited by the glucocorticoid antagonist RU 38486. Basal and agonist-stimulated cyclic adenosine 3',5'-monophosphate (cAMP) levels in arterial smooth muscle cells, before and after glucocorticoid treatment, were measured as an indicator of the physiological significance of the observed glucocorticoid-induced increase in β-adrenergic receptor binding. While causing no change in the basal cAMP level, treatment of arterial smooth muscle cells with 10^{-8} M dexamethasone for 24 hours increased the 10^{-4} M isoproterenol-stimulated cAMP levels. (Hypertension 12: 393-398, 1988)

KEY WORDS • β-adrenergic receptors • glucocorticoids • arterial smooth muscle cells • cyclic AMP • tissue culture

Gluocorticoids have been shown to regulate the expression of β-adrenergic receptors in a number of tissues, including human lymphocytes and granulocytes, 1, 2 rat liver, 3 and rat lung. 4 Glucocorticoids are also involved in the regulation of agonist-mediated β-adrenergic response in human neutrophils. 5, 6 Both glucocorticoids and catecholamines are involved in the regulation and maintenance of normal blood pressure. 7, 8 Synthetic glucocorticoids have been shown to augment pressor response to angiotensin II and norepinephrine, and this effect is blocked by glucocorticoid antagonists. 9 In addition, in Cushing’s syndrome, the pressor response to angiotensin II and norepinephrine is elevated. 10

Our laboratory has previously shown that cultured rat arterial smooth muscle cells (ASMCs) contain both glucocorticoid and β-adrenergic receptors. 11, 12 Therefore, to investigate the possibility that glucocorticoids may regulate the expression and responsiveness of β-adrenergic receptors of ASMCs, we measured β-adrenergic receptor binding before and after glucocorticoid treatment. To our knowledge, this report provides the first direct evidence for the hypothesis that glucocorticoids regulate β-adrenergic receptor expression and responsiveness in ASMCs.

Materials and Methods
All tissue culture supplies were obtained from Gibco Laboratories (Grand Island, NY, USA). All steroids were obtained from Steraloids (Wilton, NH, USA). Propranolol was a gift from Ayerst Laboratories (Rouses Point, NY, USA). Levo-[propyl-1,2,3H]dihydroalprenolol hydrochloride (DHA) and [3H]cyclic adenosine 3',5'-monophosphate were obtained from New England Nuclear (Boston, MA, USA). RU 28362 (11,17-dihydroxy-6-methyl-17α-[1-propynyl]androsta-1,4,6-trien-3-one) and RU 38486 (17β-hydroxy-11β-[4-dimethylaminophenyl]-17α[propynyl]-estra-4,9-diene-3-one) were generously provided by Roussel-UCLAF (Romainville, France).
France). All other chemicals were analytical grade and were obtained from Sigma (St. Louis, MO, USA).

Tissue Culture

ASMCs were grown from the explants of normotensive Wistar-Kyoto rat aorta as previously described.11 Cells were identified as ASMCs by morphological, growth, and biochemical characteristics.12 These included growth in multilayers in a typical "hillock and valley" pattern, absence of contact inhibition of growth, presence of myofibrils on electron microscopic examination, presence of a large amount of extracellular matrix, and presence of β-adrenergic receptors.12 ASMCs were grown in minimum essential medium supplemented with 10% fetal calf serum. Cells from Passage 5 to 20 were used. No significant detectable differences in the ASMC characteristics were observed in this range of passage number. Cells were subcultured after trypsinization on a weekly basis. Each plate was fed twice a week with fresh medium. Before all experiments, cells were placed in Medium 199 without serum for at least 20 hours.

Binding Assay

[3H]DHA binding to membrane fragments of ASMCs was performed using a modification of the method of Davies and Lefkowitz.1 Briefly, cells were scraped from plates and homogenized in a cup horn sonifier in 5 mM Tris buffer (pH 7.2), 1 mM EGTA, and 250 mM sucrose. Partially purified membrane was obtained by centrifuging the homogenate at 800 g for 5 minutes to remove intact cells and cell debris and then recentrifuging the supernatant at 40,000 g for 30 minutes at 4 °C. The membrane pellet was resuspended in binding buffer (50 mM Tris, 10 mM MgCl₂, pH 7.4) by gentle sonification. Protein concentration was determined by the method of Bradford.13 The average membrane protein content of each cell was 36 pg.

Binding was done in a total volume of 1 ml containing 300 μg of membrane protein, an appropriate amount of [3H]DHA with or without excess unlabeled competing ligand, and binding buffer. [3H]DHA was used in the concentration range of 0.5 to 25 × 10⁻¹⁰ M. Binding was performed at 30 °C for 20 minutes. The binding reaction was terminated by adding 14 ml of binding buffer at room temperature and by transferring the mixture on top of Whatman GF/C filters (Clifton, NJ, USA). The filters were washed with 40 ml of binding buffer (4 °C) and counted for radioactivity in 10 ml of scintillation cocktail (Biofluor, New England Nuclear).

Specific binding was measured by subtraction of nonspecific binding from total binding. Nonspecific binding was linear (r = 0.99) and was measured in the presence of 10⁻³ M isoproterenol. Maximum binding (Bₐₓₐₓ) and dissociation constant (Kᵤ) were determined by the method of Scatchard.14 The line of best fit was drawn through the data points by linear regression.

Steroid Treatment

Confluent plates were incubated in plain Medium 199 for at least 20 hours before each experiment. Then each plate was placed in Medium 199 with or without the appropriate amount of steroid. Steroids were added in a concentration ranging from 10⁻⁷ to 10⁻⁶ M. In all cases, except where otherwise specified, [3H]DHA binding to the cell membrane was measured after incubation of cells in the presence or absence of steroid for at least 24 hours. Dexamethasone and RU 28362 were chosen because of their high glucocorticoid activity and relatively low cross-reactivity with other steroid receptor systems.

cAMP Assay

cAMP assays were done by a competitive protein binding assay using cAMP-dependent protein kinase as described previously by Peterson et al.13 Cells were incubated in Medium L-15 with and without isoproterenol (10⁻⁶ M) for 5 minutes. Intercellular cAMP was extracted from cells by trichloroacetic acid extraction in 0.2 N HCl. Trichloroacetic acid was removed by anhydrous ether extraction, and the aqueous phase containing cAMP was concentrated to dryness by a Savant SPEEDVAC apparatus (Sel Associate, Houston, TX, USA). The pellet was dissolved in 0.2 ml of 0.1 M acetic buffer, pH 4.0. An aliquot was used for cAMP determination. The cAMP assay was done in a total volume of 70 μl containing 7.5 μg of protein kinase, 15 μg of bovine serum albumin, and 1 pmol of [3H]cAMP. The reaction mixture was incubated at 4 °C for 90 minutes. The binding reaction was terminated by the addition of 100 μl of chilled hydroxyapatite (1:3, w/vol) in 0.01 M phosphate buffer. After the hydroxyapatite pellet was washed twice with 0.01 M phosphate buffer, the pellet was dissolved in 0.3 N HCl and counted for radioactivity. Protein content was determined by dissolving all cells after trichloroacetic acid treatment in 0.5 N NaOH by the method of Bradford.13 Percent recovery after ether extraction was determined by adding 0.3 pmol of [3H]cAMP to each sample before ether extraction and counting an aliquot of the sample dissolved in 0.1 M acetic buffer. cAMP results are expressed as picomoles per milligram of total cell protein.

Statistical Analysis

[3H]DHA binding Bₐₓₐₓ and Kᵤ, as well as cAMP levels, are expressed as means ± SD. [3H]DHA Scatchard plots are series of single determinations at different concentrations. Each datum for the comparative experiments is the mean of duplicate determinations. Comparisons between data are reported using raw values, normalized data (experimental/control), or both. Data were tested for significance by the Student’s t or analysis of variance (ANOVA) using the CLINFO data management and analysis system (supported by the Clinical Research Center Program, Grant RR73, University of Texas...
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Medical Branch, Galveston, TX, USA). When ANOVA showed significance, data were compared at the 5% level against the same control by Dunnett’s multiple comparison test to identify which treatment resulted in a significant change. When each treatment had its own control, a paired t test was used.

Results

[3H]Dihydroalprenolol Binding to Control and Steroid-Treated Cells

Figure 1 shows a typical saturation curve and its Scatchard plot for [3H]DHA binding to the membrane fragments of ASMCs. Dose-response experiments performed to determine the lowest concentrations at which dexamethasone had an effect showed that 10^-9 M dexamethasone did not produce a consistent increase in [3H]DHA, whereas 10^-8 M dexamethasone significantly increased [3H]DHA binding compared with that in controls (72 ± 12%) measured at 2 nM [3H]DHA (n = 4, p < 0.05). There was no further increase in [3H]DHA binding with 10^-6 M dexamethasone. In all subsequent experiments, unless otherwise specified, 10^-6 M dexamethasone was used to ensure full occupancy of all steroid receptors. To determine the optimal incubation time for ASMCs in the presence of steroids, cells were incubated with dexamethasone for various periods ranging from 4 to 72 hours (Table 1). Dexamethasone resulted in a significant increase in binding after approximately 20 hours of treatment, but not after 4 to 15 hours. There was no further significant increase in [3H]DHA binding with 10^-6 M dexamethasone. In all subsequent experiments, unless otherwise specified, 10^-6 M dexamethasone was used to ensure full occupancy of all steroid receptors.

Scatchard analyses were performed on [3H]DHA binding at different concentrations before and after dexamethasone treatment (10^-8 and 10^-6 M) to determine if the observed increase in binding of [3H]DHA measured at 2 nM was due to a change in the affinity or the number of binding sites. Treatment of ASMCs with dexamethasone resulted in an increase in the Bmax for [3H]DHA binding (Figure 2). The results of four [3H]DHA binding experiments indicate that dexamethasone did not change the Kd (0.60 ± 0.08 nM for control and 0.89 ± 0.32 nM for dexamethasone-treated ASMCs), but it significantly increased the Bmax (2.17 ± 0.78-fold, p < 0.05). The increase in receptor number observed in ASMCs treated with glucocorticoids was not due to changes in cell protein as determined by measurement of DNA/membrane protein before and after steroid treatment (1.89 ± 0.10 for control and 2.23 ± 0.20 for dexamethasone-treated ASMCs).

To determine if the effect of glucocorticoids required protein synthesis, ASMCs were treated with plain medium and medium containing cycloheximide (10 µg/ml) alone, dexamethasone (10^-6) plus cycloheximide, or dexamethasone alone. The amount of [3H]DHA binding was compared for each condition, using ANOVA. Cell viability after cycloheximide treatment was checked microscopically.

Table 1. Influence of Incubation Time on the Stimulation of [3H]Dihydroalprenolol Binding by 10^-6 M Dexamethasone

<table>
<thead>
<tr>
<th>Time of incubation (hr)</th>
<th>Dexamethasone (% of control)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (n = 3)</td>
<td>83 ± 2</td>
<td>81-85</td>
</tr>
<tr>
<td>15 (n = 3)</td>
<td>105 ± 3</td>
<td>103-108</td>
</tr>
<tr>
<td>20 (n = 7)</td>
<td>140 ± 27*</td>
<td>115-199</td>
</tr>
<tr>
<td>48 (n = 3)</td>
<td>200 ± 78*</td>
<td>120-278</td>
</tr>
<tr>
<td>72 (n = 4)</td>
<td>176 ± 28*</td>
<td>130-195</td>
</tr>
</tbody>
</table>

Values are means ± SD.

*p < 0.02, compared with respective controls (by ANOVA).

Figure 2. Effect of dexamethasone on [3H]dihydroalprenolol binding to arterial smooth muscle cell membrane. Cells were treated either with plain medium (control, •) or with medium containing 10^-6 M dexamethasone (○) for 24 hours. Binding was done to 300 µg of membrane protein incubated at 30°C for 20 minutes. Scatchard plots were analyzed using linear regression (r = -0.98 for control and -0.99 for dexamethasone). The maximum binding for controls was 54.7 fmol/mg membrane protein with a Kd of 0.30 nM, and the maximum binding for dexamethasone-treated cells was 90.1 fmol/mg membrane protein with a Kd of 0.34 nM.

Figure 1. A typical binding curve (A) and its Scatchard plot (B) for [3H]dihydroalprenolol binding to arterial smooth muscle cell membrane. Binding was performed to 300 µg of membrane protein incubated at 30°C for 20 minutes. Linear regression was used to draw the line of best fit for the Scatchard plot (r = -0.98) and revealed maximum binding of 35.67 fmol/mg membrane protein and a Kd of 0.26 nM.
TABLE 2. Inhibition of 10^{-8} M Dexamethasone Stimulation of [3H]Dihydroalprenolol Binding to the Membrane of Arterial Smooth Muscle Cells by Cycloheximide (10 \mu g/ml)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dexamethasone (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain medium (n = 6)</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Dexamethasone alone (n = 6)</td>
<td>274 ± 50*</td>
</tr>
<tr>
<td>Cycloheximide alone (n = 4)</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>Dexamethasone + cycloheximide (n = 6)</td>
<td>132 ± 20</td>
</tr>
</tbody>
</table>

Values are means ± SD.

* p < 0.001, compared with control values (by ANOVA).

and by trypan blue exclusion technique and was found to be greater than 90%. While cycloheximide alone did not alter [3H]DHA binding, the increase in [3H]DHA binding after dexamethasone (10^{-6} M) treatment was significantly inhibited (Table 2). This observation, in combination with the long incubation period required for the steroid effect, indicates that the glucocorticoid effect requires protein synthesis.

To demonstrate that the dexamethasone effect was mediated through the glucocorticoid receptor, ASMCs were treated with plain medium alone (control) or with plain medium containing dexamethasone (10^{-8} M) or dexamethasone (10^{-6} M) plus the glucocorticoid antagonist RU 38486 (10^{-6} M). While RU 38486 alone had no effect on [3H]DHA binding, it significantly inhibited the dexamethasone effect (Figure 3).

To determine if other glucocorticoids showed a similar effect, the glucocorticoid agonist RU 28362 was used. RU 28362 (10^{-8} M) treatment of ASMCs increased the [3H]DHA binding (measured at 2 \times 10^{-9} M) significantly (62 ± 14\%; n = 4, p < 0.05). Treatment of ASMCs with RU 28362 (10^{-6} M) resulted in a Scatchard plot that was shifted to the right, indicating increased binding sites (Figure 4).

**Effect of Steroid Treatment on Isoproterenol-Induced cAMP Production**

To establish that changes in [3H]DHA binding after steroid treatment translate into a change in physiological response, basal and agonist-stimulated cellular cAMP content were measured before and after dexamethasone (10^{-6} M) treatment of ASMCs. While the basal cAMP level was not changed by steroid treatment, the 10^{-6} M isoproterenol-stimulated cAMP level of dexamethasone-treated ASMCs was significantly higher than that of their respective control (Table 3).

**Discussion**

Catecholamines influence vascular resistance through their action on specific adrenergic receptors of ASMCs.\(^7\) Stimulation of \(\alpha\)-adrenergic receptors of ASMCs produces vasoconstriction. Stimulation of \(\beta\)-adrenergic receptors causes vasodilation. \(\beta\)-Adrenergic responses are mediated through production of cAMP, which in turn activates protein kinase. Subsequently, protein kinase activation causes a cascade of events that produce inactivation of myosin kinase by phosphorylation, thus preventing vascular muscle contraction and resulting in vessel relaxation or vasodilation. It has been postulated that glucocorticoids may have a direct effect on vascular tissue reactivity and that this effect may occur through glucocorticoid-induced enhancement of cAMP production.
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of adrenergic receptor responsiveness to catecholamines. This hypothesis has been supported by the observation that ASMCs have glucocorticoid receptors and that glucocorticoid treatment enhances vascular responses to catecholamines.

Others have shown that glucocorticoids can modulate β-adrenergic receptor number and response in a number of different tissues. Davies and Lefkowitz have examined the effect of cortisone treatment on the β-adrenergic receptors of human polymorphonuclear and mononuclear leukocytes. Their results showed that, 4 hours after administration of cortisone, there was a 40% increase in the number of β-adrenergic receptors in polymorphonuclear leukocytes while the receptor number was reduced by 40% in mononuclear leukocytes. Isoproterenol-induced cAMP production in steroid-treated polymorphonuclear leukocytes was statistically higher than that in controls, while no difference was found in glucocorticoid-treated mononuclear leukocytes compared with values in controls. Mano et al. have shown a 79% increase in β-adrenergic receptor number in rat lung after hydrocortisone (a nonphysiological glucocorticoid in rat) treatment. Sundaresan and Banerjee examined the effect of hydrocortisone treatment on the β-adrenergic receptors of liver and heart in thyroidectomized rats. While there was a decrease in β-adrenergic receptor response in the liver, no change was detected in the heart. Therefore, β-adrenergic receptors are modulated differently in different tissues by glucocorticoids. To our knowledge, no previous study has documented this modulation or its magnitude in ASMCs.

In this study we found an increase in β-adrenergic receptor number induced by two highly specific synthetic glucocorticoids in ASMCs after 24 to 72 hours of incubation. The glucocorticoid treatment of ASMCs also resulted in enhanced agonist-mediated cAMP production by ASMCs. The simplest explanation for the enhanced cAMP response is the increased receptor number, but other enzymes and regulatory proteins involved in production and maintenance of cAMP levels, such as G-regulatory proteins, adenylyl cyclase, and phosphodiesterase, may also be affected by glucocorticoids. Our study cannot rule out any of these other possibilities.

With respect to these findings, steroids with glucocorticoid activity regulate the expression of β-adrenergic receptors of rat ASMCs in a similar fashion to the β-adrenergic receptors found in the rat lung and human neutrophils, but differently from those in rat liver. The increased β-adrenergic receptor number and response due to glucocorticoid treatment may result in increased potential for vasodilation and, ultimately, lower blood pressure. Indeed, using pure glucocorticoid excess, Hall et al. found lower mean arterial pressure in methylprednisolone-treated dogs compared with values in controls.

It is difficult to explain the effects of glucocorticoids on blood pressure in whole animals or humans based on our results in the present study. This study employed a tissue culture model to explore the peripheral vascular effects of pure synthetic glucocorticoids on β-adrenergic receptor binding characteristics. While our results have demonstrated that the potential for vasodilation through β-adrenergic receptor binding and isoproterenol-induced cAMP response is increased after glucocorticoid treatment of ASMCs, we can only speculate as to the effect of endogenous glucocorticoids in vivo. However, if endogenous glucocorticoids act through the glucocorticoid receptor, as do synthetic glucocorticoids, it is very likely that they too show similar effects on β-adrenergic receptors of ASMCs. In fact, the synthetic glucocorticoid methylprednisolone has been shown to have renal vasodilator effects that may contribute to glomerular injury in treated rats with renal ablation.

Since peripheral vascular regulation of blood pressure is controlled mostly by the small resistance arteries rather than by large arteries such as the aorta, which is not considered a resistance vessel, we must exercise caution in extrapolating our results. However, it is unlikely that the β-adrenergic receptor gene in smooth muscle cells from aorta differs from the β-adrenergic receptor gene in smooth muscle cells from other vessels. Experiments using larger animals will be necessary to compare cells from various vessel types.

Further studies are necessary to determine the direct effect of pure glucocorticoids on other ASMC receptors (i.e., angiotensin or α-adrenergic receptors, which cause vasoconstriction). The responses of these receptors may also be enhanced after treatment of ASMC with glucocorticoids. The advantage of the ASMC culture system used in this study is that the effect of a variety of hormones and drugs on the peripheral vascular cells can be determined individually. Once a better understanding of the peripheral vascular cells is obtained, the results obtained in
whole animals after treatment with these hypertensive hormones and drugs can be better interpreted.

In summary, the present study has shown that pure synthetic glucocorticoids have a direct action on the peripheral vascular tissue mediated through increased β-adrenergic receptor number and agonist-mediated response. The model employed by this study can be used to determine the effects of other vasoactive compounds on the ASMC.

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