Effect of glucocorticoids on $\alpha_1$-adrenergic receptor binding in rat vascular smooth muscle

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ABSTRACT

Glucocorticoids are known to have marked effects on blood pressure regulation, predominantly through altering cardiovascular sensitivity to noradrenaline. However, the molecular mechanisms underlying this action remain unclear. As part of our studies into these we have measured $\alpha_1$-adrenergic receptor binding using the ligand [3H]prazosin in plasma membrane fractions of aortas prepared from control, adrenalectomized and dexamethasone-treated adrenalectomized rats. In controls there were $50 \pm 8$ (S.E.M.; $n=6$) fmol $\alpha_1$-adrenergic receptors/mg membrane protein ($B_{\text{max}}$) with a dissociation constant ($K_d$) of $0.52 \pm 0.10$ nM ($n=6$). Adrenalectomy 8 days before tissue preparation caused a 40% decrease in $B_{\text{max}}$ and a 60% decrease in $K_d$. Dexamethasone replacement after adrenalectomy returned these values close to those of controls. Noradrenaline competed for the [3H]prazosin-binding sites. Computer analysis by a non-linear curve-fitting program (LIGAND) showed that noradrenaline binding was to a heterogeneous population of high- and low-affinity receptors with $K_d$ values of $1.87 \pm 0.73 \mu M$ and $0.48 \pm 0.12 \mu M$ ($n=5$) respectively. Guanosine thiotriphosphate (GTP[S]) caused the conversion of high-affinity to low-affinity binding, consistent with the model of the high-affinity sites being coupled to a G protein. After adrenalectomy, noradrenaline binding was to a homogeneous population of low-affinity receptors; hence, the effect of GTP[S] was no longer apparent, suggesting that under these conditions the $\alpha_1$-adrenergic receptors were unable to couple to a G protein. The two-site model of binding and GTP[S] effect was returned by dexamethasone treatment. These data provide evidence that glucocorticoids not only modulate the number of $\alpha_1$-adrenergic receptors on vascular smooth muscle, but also cause disruptions in receptor–G protein coupling. This may be an important mechanism by which glucocorticoids exert their effect on cardiovascular sensitivity.

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INTRODUCTION

The adrenal steroids are known to play an important role in the regulation of blood pressure (Martin, 1985). Mineralocorticoids have established effects on blood pressure regulation through their action on salt and water balance, whereas a major action of glucocorticoids appears to be modulation of vascular smooth muscle sensitivity to noradrenaline (Yagil, Koreen & Krakoff, 1986; Frohlich, 1987; Grunfeld & Eloy, 1987; Yagil & Krakoff, 1988; Darlington, Kaship, Keil & Dallman, 1989; Sudhir, Jennings, Esler et al. 1989). The molecular mechanisms underlying the action of glucocorticoids are, however, still unclear.

The contractile state of vascular smooth muscle is regulated by the sympathetic nervous system which acts to modulate the free intracellular $Ca^{2+}$ concentration, largely through activation of $\alpha_1$-adrenergic receptors (Bulbring & Tomita, 1987). Noradrenaline released from nerve terminals binds to these receptors causing them to couple to a guanine nucleotide-binding protein (G protein) which, in turn, activates membrane-bound phospholipase C (Fain, Wallace & Wojcikiewicz, 1988). This enzyme catalyses the release of second messengers, primarily diacylglycerol and inositol trisphosphate (Berridge, 1987), which act to modulate intracellular $Ca^{2+}$ concentrations and hence contractility (Bulbring & Tomita, 1987). A principle mechanism for the regulation of this pathway is the modulation of receptor number and affinity (Davies & Lefkowitz, 1984). Hence, steroid-induced alterations of vascular smooth muscle sensitivity could derive from changes in the...
interaction between ligand and vascular smooth muscle receptor sites.

Such a mechanism has been shown to exist for the regulation of β-adrenergic receptors in cultured vascular smooth muscle cells. In this system there was an increase in the number of receptors, but no change in affinity, accompanying prolonged incubation with increasing concentrations of synthetic glucocorticoids (Jazayeri & Meyer, 1988). The effects of steroids on the α1-adrenergic receptor in vascular smooth muscle, however, are still unclear. Therefore, the aim of the present study was to evaluate the effects of glucocorticoids on α1-adrenergic receptor binding in vascular smooth muscle of the rat.

MATERIALS AND METHODS

Animals

Control and bilaterally adrenalectomized male Sprague–Dawley rats (200–225 g) were obtained from Harlan Olac (Bicester, Oxon, U.K.). Adrenalectomized rats were maintained on 0.9% (w/v) NaCl in their drinking water. All animals were allowed free access to standard rat diet and water. Completeness of adrenalectomy was ascertained by measuring depletion of plasma corticosterone as previously described (Edwards, Hansell & Jones, 1986) and post-mortem examination of the kidneys.

Steroid treatment

Adrenalectomized animals receiving glucocorticoid treatment were given daily s.c. injections of dexamethasone phosphate (500 µg/kg body weight in saline) starting 24 h after the operation. The final dose was given 3 h before the rats were killed.

Tissue preparation

Animals were killed by cervical dislocation following a blow to the head. Adrenalectomized animals were killed 8 days after the operation. The thoracic and abdominal aorta was quickly removed to ice-cold phosphate-buffered saline and cleaned of adherent fat and connective tissue. The aorta was blotted dry, rapidly frozen and stored at −50 °C until required for assay.

For each binding experiment a crude plasma membrane fraction was prepared from six aortas. The tissue was finely minced and then homogenized in 10 ml ice-cold 10 mM Tris–HCl (pH 8.0 at 4 °C) with two 30-s bursts of an Ultra-Turrax TP 18/10 homogenizer (Scientific Instrument Centre, London, U.K.) interspersed with a 90-s period of cooling. After the addition of an equal volume of ice-cold 10 mM Tris–HCl (pH 7.5 at 4 °C) containing 500 mM sucrose and 2 mM EGTA, the crude homogenate was centrifuged at 250 g for 10 min at 4 °C. The resulting pellet was extracted twice more by homogenization and centrifugation. The pooled supernatants were filtered through four layers of cheesecloth, and the crude plasma membrane fraction was isolated by centrifugation at 50 000 g for 30 min at 4 °C. The surface of the pellet was carefully washed with 1–2 ml assay buffer (25 mM glycyglycine plus 10 mM MgCl2, pH 7-6 at 30 °C) before resuspension in assay buffer to a protein concentration of 0.5–1.0 mg/ml. Protein was determined by the BCA assay kit of Pierce Chemical Company (Pierce & Warriner (UK) Ltd, Chester, Cheshire, U.K.) using bovine serum albumin as standard.

Binding assay

Binding of [3H]prazosin to α1-adrenergic receptors was determined using a rapid filtration method. Saturation binding studies, performed in triplicate, were initiated by the addition of 200 µl crude plasma membrane preparation to 200 µl prewarmed assay buffer containing various concentrations of [3H]prazosin (0.05–5 nM). A parallel set of tubes, in triplicate, also contained 10 µM phenolamine to determine non-specific binding. Incubations were continued for 30 min at 30 °C with shaking at 50 cycles per minute. Binding was terminated by the addition of 5 ml ice-cold wash buffer (50 mM Tris–HCl plus 10 mM MgCl2, pH 7-5 at 4 °C) and rapid filtration through Whatman GF/B glass-fibre filter paper using a Brandel M 24R cell harvester (Semat Technical (UK) Ltd, St Albans, Herts, U.K.). Filter paper was soaked in 2% (v/v) polyethyleneimine for 10 min before use to reduce filter binding. Tubes and filters were washed with a further 2 × 5 ml ice-cold wash buffer and the filters removed to plastic vials. Aquasol (10 ml) (NEN/Du Pont (UK) Ltd, Stevenage, Herts, U.K.) was added to the vials which were allowed to stand overnight in the dark before liquid scintillation counting at an efficiency of 42–48%. For competition studies, binding was carried out as above with a fixed concentration of [3H]prazosin (approximately 0.5 nM) and various concentrations of noradrenaline (10 nM–100 nM). The reaction mixture also contained 0.1% (w/v) ascorbic acid and 10 µM pargyline to inhibit the oxidation of noradrenaline. These additions did not affect the specific binding of [3H]prazosin. Total binding was obtained in the absence of noradrenaline, and non-specific binding in the presence of 10 µM phenolamine.
To study the effects of non-hydrolysable analogues of GTP on noradrenaline binding, competition studies were performed as above but in the presence of 100 μM guanosine thiotriphosphate (GTP[S]).

Analysis of data

All binding data were analysed by the computerized, non-linear curve-fitting program LIGAND (McPherson, 1983). Results are expressed as the mean ± s.e.m. for the given number of observations (n). Statistical differences between means were calculated by one-way analysis of variance (ANOVA). Values were considered statistically significant when P<0.05.

Materials

[^3]H]Prazosin ([7-methoxy-[^3]H]prazosin; 82 Ci/mmol) was obtained from NEN/Du Pont (UK) Ltd, noradrenaline and GTP[S] (guanosine-5′-O-(3-thiotriphosphate) tetralithium salt) were from Sigma Chemical Company (Poole, Dorset, U.K.) and dexamethasone sodium phosphate was from Organon Laboratories (Cambridge, Cambs, U.K.). Phentolamine was a kind gift from Ciba Laboratories (Horsham, Sussex, U.K.). All other chemicals were of the highest grade commercially available.

RESULTS

Characteristics of receptor binding

Prazosin is a well-established ligand which binds almost exclusively to α1-adrenergic receptors. In our assay system, antagonists of the adrenergic receptor competed for prazosin binding with the following order of potency: prazosin > phentolamine > yohimbine (results not shown). In addition, agonists of the adrenergic receptor competed for prazosin binding in the order: noradrenaline > adrenaline > phenylephrine (results not shown). These results are consistent with[^3]H]prazosin binding to α1-adrenergic receptors in this assay system (Ruffolo, 1985). Binding of[^3]H]prazosin over a wide concentration range (0.05–5 nm) was saturable, reversible and to a single class of receptors when non-specific binding was assessed with 10 μM phentolamine. Non-specific binding was approximately 50% of the total binding when the concentration of[^3]H]prazosin was 1 nm. Figure 1 shows a typical saturation curve and Scatchard transformation of the data by LIGAND; the results are representative of those obtained from six groups of normal rats.

Receptor number and affinity

Figure 2 shows the Scatchard transformation of saturation curves which are representative of data from control, adrenalectomized and treated animals. In normal animals, the total number of α1-adrenergic receptors in the plasma membrane (B<sub>max</sub>) was...
50 ± 8 fmol receptors/mg membrane protein (n = 6). Adrenalectomy caused a 40% decrease in B_{max} (30 ± 2 fmol/mg; n = 5) which was significantly (P < 0.05) different from controls. Treatment with dexamethasone, after adrenalectomy, was used to evaluate the specific effects of glucocorticoids on ligand binding. After dexamethasone treatment there was a 53% increase in B_{max} (46 ± 13 fmol/mg; n = 5) over the adrenalectomized values. This value was not significantly different from either the control or adrenalectomized values. The effects of these treatments on the dissociation constant (K_d) of the receptors was also assessed. In control animals K_d was 0.52 ± 0.10 nM (n = 6) and steroid depletion caused a 60% decrease (P < 0.05) in K_d (hence an increase in apparent affinity) to 0.21 ± 0.04 nM (n = 5). This value was also significantly (P < 0.05) different from adrenalectomized animals treated with dexamethasone. However, the K_d value (0.71 ± 0.14 nM; n = 5) of adrenalectomized animals treated with dexamethasone was not significantly different from control levels.

**Noradrenaline binding**

Noradrenaline competed for [3H]prazosin-binding sites on rat aorta plasma membranes. The competition curves were shallow (see Fig. 3 for a representative curve) and computer analysis by LIGAND showed that the data was statistically better fitted by a curve for a two-site model (ANOVA; P < 0.05 compared with a one-site fit) with high- and low-affinity receptors (Table 1). In the presence of 100 μM GTP[S] there was a steepening of the competition curve, such that a one-site model was the most appropriate fit. LIGAND analysis indicated that the high-affinity receptors had been converted to the low-affinity form, so that in the presence of GTP[S], noradrenaline binding was to a homogeneous population of low-affinity α_{1}-adrenergic receptors. These results are consistent with the hypothesis that high-affinity receptors are those coupled to a G protein, whereas low-affinity receptors are uncoupled (Milligan, 1988).

The effect of adrenalectomy on noradrenaline binding in rat aorta is shown in Fig. 4. In the absence of GTP[S], LIGAND analysis showed that the curve was best fitted by a one-site model, indicating a homogeneous population of receptors. In the presence of GTP[S] the curve was superimposable upon the curve representing binding in the absence of GTP[S], indicating the lack of effect of GTP[S]. Further analysis of this data (Table 1) revealed that noradrenaline bound to a single class of low-affinity receptors in the absence of GTP[S]; hence, addition of GTP[S] could have no further effect.

The noradrenaline competition curve from adrenalectomized animals after treatment with dexamethasone is shown in Fig. 5, which demonstrates that the two-site model of noradrenaline binding is restored in the absence of GTP[S]. LIGAND analysis (Table 1) revealed that dexamethasone treatment caused a return of the heterogeneous population of high- and low-affinity receptors. As with the control animals, GTP[S] caused a shift of the high-affinity receptors to a low-affinity form, indicating that heterogeneous binding was restored by dexamethasone.

**DISCUSSION**

It is now well established that adrenal steroids have marked effects on blood pressure regulation. Impaired vascular sensitivity to adrenal steroids is a major factor contributing to the abnormal regulation of arterial pressure in the absence of adrenal steroid secretion. Thus, adrenalectomy is associated with a reduced cardiovascular response to pressor agents (Yagil et al. 1986; Yagil & Krakoff, 1988; Darlington et al. 1989). These effects can be reversed by glucocorticoid treatment alone (Yagil & Krakoff, 1988). However, the molecular mechanisms in-
TABLE 1. Effect of guanosine triphosphate (GTP[S]) on binding of noradrenaline to vascular smooth muscle from intact and adrenalectomized (ADX) rats and from adrenalectomized rats treated with dexamethasone. Values are means ± S.E.M. for five experiments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_d1$ (µM)</th>
<th>$K_d2$(µM)</th>
<th>$B_{max}1$ (%)</th>
<th>$B_{max}2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact control</td>
<td>1.87 ± 0.73</td>
<td>0.48 ± 0.12</td>
<td>28.8 ± 3.8</td>
<td>71.2 ± 3.8</td>
</tr>
<tr>
<td>Intact + GTP[S]</td>
<td>0.31 ± 0.14</td>
<td>0.05 ± 0.03</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ADX</td>
<td>0.05 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>38.9 ± 9.1</td>
<td>62.1 ± 9.1</td>
</tr>
<tr>
<td>ADX + dexamethasone</td>
<td>1.14 ± 0.27</td>
<td>4.00 ± 0.10</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ADX + dexamethasone + GTP[S]</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>38.9 ± 9.1</td>
<td>62.1 ± 9.1</td>
</tr>
</tbody>
</table>

The dissociation constants ($K_d$) and the number of receptors ($B_{max}$) were calculated from competition curves by the non-linear curve-fitting program LIGAND. $K_d1$ and $K_d2$ represent the high-affinity and low-affinity form of the receptor respectively. $B_{max}1$ and $B_{max}2$ are the number of high-affinity and low-affinity receptors respectively, expressed as a percentage of the total number of binding sites. Each model was statistically ($P < 0.05$ by ANOVA) the best fit as determined by LIGAND.

![Graph showing prazosin binding to noradrenaline in the aorta from adrenalectomized rats.](image)

**FIGURE 4.** Inhibition of $[^3]H$prazosin binding by noradrenaline in the aorta from adrenalectomized rats. Binding studies were performed in the absence (●) and presence (▲) of guanosine triphosphate (GTP[S]) as described in Materials and Methods. Data are from one representative experiment performed in triplicate. Binding of noradrenaline was similar in both the absence and presence of GTP[S]. Both curves were best-fitted statistically (ANOVA; $P < 0.05$) by a one-site model, rather than a two-site model, by the non-linear curve-fitting program LIGAND.

Involvement in glucocorticoid modulation of vascular sensitivity are still unclear.

The first step in the action of noradrenaline on vascular smooth muscle, at the level of the plasma membrane, is binding to post-junctional $\alpha_1$-adrenergic receptors (Bulbring & Tomita, 1987). Few studies, however, using ligand binding analysis, have examined the possibility that decreased sensitivity after adrenalectomy derives from changes in the interaction of ligand with receptor. Our study is one of the first to demonstrate that there is a significant decrease in the number of $\alpha_1$-adrenergic receptors in rat aorta following adrenalectomy. Changes in receptor number provide one possible mechanism by which adrenal steroids modulate vascular sensitivity. Our study also shows, however, that there is an increased affinity of the receptor concomitant with the fall in number. Receptor affinity is a second important determinant of sensitivity to noradrenaline in vascular smooth muscle (Colucci, Gimbrone & Alexander, 1984; Bevan, Oriowo & Bevan, 1986), tissue sensitivity at the level of the receptor being a product of both receptor reserve and affinity (Ruffolo, 1982). Thus, it is important to note that the countervailing changes in binding properties of the $\alpha_1$-adrenergic receptor in this study, i.e. increased affinity accompanying down-regulation, could easily mask changes in the functional properties of rat aorta. Hence, it is tempting to speculate that the increase in affinity is a mechanism by which smooth muscle cells offset the decrease in receptor reserve.

As a means of testing the effects of glucocorticoids we chose to use dexamethasone treatment after adrenalectomy. Yagil & Krakoff (1988) have previously shown that dexamethasone alone, but not
Aldosterone, can return to control values the pressor responses to noradrenaline in adrenalectomized rats. It has also been shown that aorta cells have glucocorticoid receptors (Jazayeri & Meyer, 1988) and that glucocorticoid treatment enhances vascular responses to noradrenaline (Grunfeld & Eloy, 1987). In addition, mineralocorticoids have been shown to have little effect on vascular smooth muscle at the level of the receptor. Smith, Jones, Bylund & Jones (1987) found that excess aldosterone had no effect on aorta $\alpha_1$-adrenergic receptor number, affinity or type, and Meggs, Stitzel, Ben-Ari et al. (1988) showed that the up-regulation of vascular $\alpha_1$-adrenergic receptors in response to deoxycorticosterone acetate did not correlate with increased sensitivity. In our study, dexamethasone treatment after adrenalectomy returned the $K_d$ and $B_{max}$ of the receptors close to control values. These findings support the hypothesis that glucocorticoids modulate cardiovascular sensitivity by altering the $\alpha_1$-adrenergic receptor reserve in vascular smooth muscle.

A finding common to many studies of the effects of steroids on $\alpha_1$-adrenoceptor responses has been the lack of correlation between tissue response and changes at the level of the receptor, implying involvement of further membrane factors. Under physiological conditions, vascular smooth muscle activity is regulated by changes in the intracellular free Ca$^{2+}$ concentration, largely through activation of $\alpha_1$-adrenergic receptors (Bulbring & Tomita, 1987). Noradrenaline-bound receptors are thought to couple to membrane-bound G proteins, causing the release of a G protein subunit which, in turn, activates membrane-bound phospholipase C (Gilman, 1987; Fain et al. 1988). This enzyme catalyses the release of inositol trisphosphate and diacylglycerol from phosphatidylinositol bisphosphate (Berridge, 1987), which act as intracellular second messengers to cause Ca$^{2+}$ release and Ca$^{2+}$ flux through the membrane (Bulbring & Tomita, 1987). Hence, steroid effects on sensitivity to noradrenaline at the level of the smooth muscle membrane may also be exerted through modulation of either G protein, phospholipase C or both. Recent evidence suggests that this transmembrane signalling pathway is a potential target for the action of steroids. In cardiovascular tissues from steroid-treated rats, there is both an increased production of inositol phosphate second messengers (Eid & De Champlain, 1988) and an increased K$^+$ efflux (Smith et al. 1987) in response to noradrenaline, despite the absence of a change in the $\alpha_1$-adrenergic receptor. Moreover, the steroid-induced increase in K$^+$ efflux was associated with altered coupling between the receptor and production of second messengers (Jones, Geisbuhler, Shukla & Smith, 1988). Similar observations have been made from studies on liver membranes prepared from adrenalectomized animals (Guellaen, Yates-Aggerbeck, Vauquelin et al. 1978; Chan, Blackmore, Steiner & Exton, 1979; El-Refai & Chan, 1986; Freudenrich & Borle, 1988). The implication is, therefore, that adrenal steroid depletion is associated with disruption of the post-receptor transmembrane signalling pathway.

Clearly, in the present experiments, changes in the transmembrane signalling pathway may have accompanied adrenalectomy and steroid replacement. As part of our initial studies of such an action we examined the coupling of the receptor to G protein using noradrenaline competition for $[^3]$H]prazosin binding in the absence or presence of GTP[S]. In control animals, GTP[S] caused a shift in noradrenaline binding such that the heterologous population of high- and low-affinity receptors was converted to a homogeneous population of the low-affinity type. These data are consistent with a model whereby in the absence of GTP[S], noradrenaline interacts with receptors in intimate association with a G protein with higher affinity than those receptors alone (Milligan, 1988). After adrenalectomy the effect of GTP[S] was no longer seen, since in the absence of GTP[S] all receptors were of the low-

**Figure 5.** Inhibition of $[^3]$H]prazosin binding by noradrenaline in the aorta from dexamethasone-treated adrenalectomized rats. Binding studies were performed in the absence (●) and presence (▲) of guanosine thiotriphosphate (GTP[S]) as described in Materials and Methods. Data are from one representative experiment performed in triplicate. In the absence of GTP[S], data points were best fitted by a curve for a two-site model by the non-linear curve-fitting program LIGAND (ANOVA; $P < 0.05$ compared with a one-site model), indicating the return of high- and low-affinity binding. The effect of GTP[S] was similar to that in intact animal control experiments.
affinity form and therefore presumably all uncoupled from G protein. The effect of GTP[S], and hence coupling, was restored after treatment with dexamethasone. These results indicate that glucocorticoids are required for proper maintenance of receptor–G protein coupling and that alterations in this coupling may be an alternative mechanism by which steroids control vascular sensitivity.

These experiments give no indication as to how steroids control G protein coupling. There is, however, increasing evidence to suggest that modulation of the steady-state amount of G protein available for coupling is one mechanism by which transmembrane signalling may be controlled. Gs and Gi are the G proteins which couple receptors to the stimulation and inhibition of adenylate cyclase respectively (Gilman, 1987). Adrenalectomy has been shown to decrease the concentration of Gs and at the same time to increase the concentration of Gi in plasma membranes prepared from liver (Garcia-Sainz, Huerta-Bahena & Malbon, 1989). Similarly, Ros, Northup & Malbon (1989) have shown a decrease in Gs in adipocytes from adrenalectomized rats which could be reversed by treatment with dexamethasone. At least part of the action of glucocorticoids on Gs seems to be at the level of increased expression of mRNA (Chang & Bourne, 1987; Saito, Guitart, Hayward et al. 1989).

In conclusion, our results provide further evidence that a major effect of glucocorticoids on vascular smooth muscle is to alter the number of α₁-adrenergic receptors on the cell membrane. This may be one mechanism by which steroids modulate vascular sensitivity. In addition, we have provided new evidence to suggest that glucocorticoids also have a profound effect on coupling of the α₁-adrenergic receptor to a G protein. Since it is clear that the effects of steroids on vascular smooth muscle cannot easily be explained by changes in receptor number, modulation of the coupling pathway, particularly at the level of the G protein, provides an attractive alternative mechanism.

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