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Glucocorticoids but not Mineralocorticoids Modulate Endothelin-1 and Angiotensin II Binding in SHR Vascular Smooth Muscle Cells

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Both glucocorticoids and mineralocorticoids are involved in circulatory homeostasis and blood pressure control. In recent years direct effects of both steroid classes on vascular smooth muscle cells (VSMC) have been reported. We have thus examined the effects of RU 28362, a pure glucocorticoid agonist, and aldosterone, the physiologic mineralocorticoid, on the binding to VSMC from spontaneously hypertensive rats (SHR) of two key vasoactive peptides, endothelin-1 and angiotensin II. Binding of angiotensin II rose, and that of endothelin-1 declined, in a time- and dose-dependent fashion with maximal effects observed at 24 h and half-maximal effects for each at 2–3 nM RU 28362. Scatchard analysis showed that for both endothelin-1 and angiotensin II, RU 28362 alters receptor number but not affinity; competition studies with receptor-selective ligands (BQ123, S6C, DuP753 and PD123319) show that glucocorticoids specifically elevate (X2) AT-1 receptors and specifically lower (to ~30%) levels of ET_A receptors. Treatment of VSMC with the antiglucocorticoid RU 38486 reversed the effect of glucocorticoids on endothelin-1 and angiotensin II binding, confirming the Type II (glucocorticoid) receptor mediated effect of the glucocorticoids. Aldosterone (100 nM) also lowers endothelin-1 binding and increases angiotensin II binding in VSMC; that this effect reflects aldosterone occupancy of classical glucocorticoid receptors is shown by the blockade of the aldosterone effect by an equal concentration (100 nM) of RU 38486—i.e. there is no evidence for an action of aldosterone via mineralocorticoid receptors. We interpret our results as evidence for a complex modulation of receptors for vasoactive peptides in VSMC by glucocorticoid but not mineralocorticoid hormones.

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INTRODUCTION

Both glucocorticoids and mineralocorticoids are involved in circulatory homeostasis, and excess of either class of steroids may lead to hypertension [1, 2]. Whereas mineralocorticoids appear to have their effects both peripherally and centrally, in terms of blood pressure elevation [3], the primary effect of glucocorticoids is to alter vascular reactivity [4]. By two-dimensional gel electrophoresis of proteins expressed by cultured vascular smooth muscle cells (VSMC), glucocorticoids have been shown to alter the synthesis of 12 proteins (the glucocorticoid domain), which are either increased or decreased by steroid treatment [5]; in these studies no unique "mineralocorticoid domain" of protein synthesis could be found.

Glucocorticoids have also been reported to increase levels of both β-adrenergic [6] and α_1-adrenergic receptors [7] in rat VSMC cells. Effects on the second messenger systems have similarly been reported in VSMC, including increases in calcium uptake and [^{3}H]dihydropyridine binding in A7r5 VSMC [8], and of IP_3 formation in the presence of angiotensin II, vasopressin or endothelin-1 in rat VSMC [9, 10]. Glucocorticoids have similarly been shown to increase VSMC cAMP formation in the presence of dopamine [11] and prostaglandin E_1 [12]. There are, in addition, conflicting reports on the effects of glucocorticoids on G protein levels in rat VSMC and aorta [13, 14]. Glucocorticoids have also been reported to potentiate

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Na^+–H^+ exchange in VSMC [15], to decrease prostaglandin release in rabbit coronary microvessel endothelium [16], and to lower nitric oxide synthetase activity in rings of rat thoracic aorta [17].

Recently, Nambi *et al.* [18] have reported a decrease in endothelin-1 binding in VSMC by treatment with pM concentrations of dexamethasone. Endothelin is a very potent vasoconstrictor [19], so that at first sight these results may seem surprising, since most of the glucocorticoid effects reported on vascular smooth muscle cells favour vasoconstriction, consistent with the hypertensive effects of glucocorticoid excess *in vivo*. In this study we report the effects of both glucocorticoids and mineralocorticoids in VSMC in culture on the binding of two potent vasoconstrictors, endothelin-1 and angiotensin II.

METHODS

Male spontaneously hypertensive rats (SHR) (~12 weeks old) were from a colony maintained at the Baker Medical Research Institute (Melbourne, Australia). Fetal calf serum and penicillin/streptomycin were from Commonwealth Serum Laboratories (Melbourne, Australia). DMEM was from FLOW Laboratories (Melbourne, Australia) and tissue culture dishes from Sterilin Ltd (Feltham, U.K.). Collagenase and elastase were from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). [^{125}I]endothelin and [^{125}I]angiotensin II were from Du Pont Australia Ltd (North Ride, NSW, Australia). Cortisol and aldosterone were purchased from Sigma (St Louis, MO, U.S.A.). The synthetic steroid analogues RU 28362 and RU 38486 were gifts of Roussell-Uclaf (Romainville, France). HEPES and BSA were from Boehringer Mannheim (Mannheim, Germany). The endothelin receptor A (ET_A) antagonist BQ123 and the endothelin receptor B (ET_B) agonist sarafotoxin (S6C) were purchased from Auspep (Melbourne, Australia). The angiotensin Type 1 receptor (AT-1) antagonist Losartan (DUP753) was kindly provided by Dupont Merck Pharmaceutical Co. (Wilmington, DE, U.S.A.). The angiotensin Type 2 receptor (AT-2) antagonist PD123319 was kindly provided by Parke-Davis (Ann Arbor, MI, U.S.A.).

Isolation of aortic smooth muscle cells

Primary cultures of vascular smooth muscle cells were prepared by enzymatic dispersion of aortic media from 12-week-old SHR as previously described [20]. Though clearly the aorta is not a primary resistance vessel, it is a reliable source of vascular smooth muscle cells for *in vitro* studies, and as such has been widely used in previous studies on glucocorticoid effects on vascular smooth muscle [5, 6, 9–12, 21]. In brief, thoracic aortae were dissected under sterile conditions and placed in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 60 μg/ml penicillin/streptomycin. The vessels were cleared of fat and connective tissue, cut longitudinally and then incubated at 37°C for 30 min with 3 mg/ml collagenase to remove the endothelium. The aortae were transferred to fresh 10% FCS/DMEM and the media plus intima peeled from the adventitia with watchmaker's forceps. The muscle layer was then cut into 1–2 mm strips and incubated for several hours at 37°C with collagenase and elastase (0.5 mg/aorta) in DMEM. The final cell suspension was centrifuged at 250 *g* for 3 min at room temperature, the medium was carefully aspirated and the pellet gently resuspended in fresh 10% FCS/DMEM. Cells were grown in 10% FCS/DMEM and a confluent layer obtained within 7 days in 90 mm tissue culture dishes. Examination by phase-contrast microscopy showed that the cells formed a hills and valleys pattern at confluence, a well known characteristic of VSMC in culture. Smooth muscle cell identity was verified by immunocytochemical analysis using specific antibodies for smooth muscle α-actin [22]. VSMC between passage 3–8 were used in all experiments.

Steroid treatment

For each experiment, confluent cells were passaged, plated in 24-multi well plates and grown in 10% FCS/DMEM until confluent (~250,000 cells). The cells were then deprived of serum for 24 h, the medium replaced (DMEM without serum) and steroid treatment commenced. To determine the time- and dose-dependent effects of glucocorticoids on vasoactive binding, VSMC were treated for up to 48 h with RU 28362 (100 nM) or with increasing concentrations of RU 28362 (from 0 to 100 nM) for 24 h. In parallel studies, dexamethasone at equal concentrations for the same time periods was also used, with indistinguishable results. The possibility of specific mineralocorticoid receptor mediated effects was also explored, by measuring VSMC binding after incubation for 24 h with media alone, and with aldosterone (100 nM) in the presence or absence of the antiglucocorticoid RU 38486 (100 nM). In all experiments the variation between groups in cell number at harvest was $<9\%$. All studies were in triplicate, except for Scatchard analyses which were in quadruplicate, and repeated at least once. For each study data were analysed by ANOVA, and are shown separately.

[^{125}I]endothelin-1 and [^{125}I]angiotensin II binding

Cells were washed twice in PBS after steroid treatment and media replaced with 200 μl of DMEM with 1% BSA (w/v), 20 μM HEPES and 0.01 nM [^{125}I]endothelin-1 (specific activity 2000 Ci/mmol) for endothelin-1 binding studies and 0.1 nM angiotensin II (specific activity 2200 Ci/mmol) for angiotensin II binding studies. Non-specific binding was determined for each treatment by adding 1 μM endothelin-1 or

1 μM angiotensin II to the incubation medium and was subtracted from total binding. In a typical binding experiment, total binding was 1300 and 750 cpm and non-specific binding was 250 and 150 cpm for [^{125}I]angiotensin II and [^{125}I]endothelin-1, respectively. For the competition studies, [^{125}I]endothelin-1 binding was determined in the presence or absence of 10 nM BQ123 or S6C, and [^{125}I]angiotensin II binding in the presence or absence of 100 nM DUP753 or PD123319. VSMC were incubated for 16 h on a rotating shaker at 4°C. Verification of tracer integrity at the end of the incubation period routinely showed [^{125}I]angiotensin II to be $\geqslant$85% intact, and [^{125}I]endothelin-1 to be $\geqslant$92% intact. Cells were then placed on ice, the incubating media aspirated, the wells washed three times with 1 ml PBS containing 1% BSA (w/v) and washed cells removed by incubation at 37°C in 0.5 ml NaOH (1 M). After 1 h the NaOH was transferred to glass tubes, the wells rinsed with an additional 0.5 ml of NaOH and the bound [^{125}I]endothelin-1 or [^{125}I]angiotensin II counted in a γ-counter.

Data analysis

Statistical analysis was by ANOVA (Stat View, Abacus, CA, U.S.A.) and Scatchard analysis by a non-linear curve fitting program (LIGAND).

RESULTS

The highly specific synthetic glucocorticoid RU 28362 at a dose of 100 nM significantly affected the binding of both angiotensin II and endothelin-1 to VSMC from SHR (Fig. 1). After an initial fall at 6 h, binding of angiotensin II increased to plateau levels of ~80% higher than control at 18–48 h. The pattern of endothelin-1 binding was in a sense the mirror image, with a fall to nadir values of ~30% of control by 24 h, followed by a return to ~70% control levels after 48 h incubation. Similar results were obtained when VSMC were incubated with dexamethasone (100 nM) rather than RU 28362 (data not shown).

For both ligands the change in binding site concentration was dose-dependent, as shown in Fig. 2. After 24 h of incubation with a range of doses of RU 28362 (0.5–100 nM), binding of angiotensin II rose, and that of endothelin-1 declined, with half-maximal effects for each ligand at steroid concentrations of 2–3 nM. Similar results were obtained when VSMC were treated with increasing concentrations of dexamethasone rather than RU 28362, with a half-maximal effect between 1–10 nM (data not shown).

That the glucocorticoid effect on angiotensin II and endothelin-1 binding represents a change in receptor concentration rather than affinity is shown by the data in Fig. 3. In both instances, the calculated slope of the Scatchard plot for high affinity binding is unaltered (K_d: 0.2 ± 0.1 nM in control cells vs 0.2 ± 0.1 nM in glucocorticoid treated cells for endothelin-1 and 1.5 ± 0.4 vs 2.4 ± 0.1 nM for angiotensin II), indicating unaltered affinity; in both instances, the major difference between the presence of steroid (RU 28362, 100 nM, 24 h) and the absence is in terms of the x-axis intercept, representing receptor concentration (B_{max}: 3.2 ± 0.9 vs 1.5 ± 0.6 × 10^3 sites/cell for endothelin-1 and 4.1 ± 1.0 vs 7.7 vs 0.2 × 10^3 sites/cell for angiotensin II). Similar results were obtained when cells were treated with 100 nM dexamethasone (data not shown).

Because RU 28362 is a highly selective Type II glucocorticoid receptor agonist, the effects of angiotensin II and endothelin-1 binding shown in Figs 1–3 clearly reflect classical glucocorticoid receptor occupancy. Whether Type I (mineralocorticoid) receptor occupancy can produce equivalent effects on binding in VSMC was studied by incubating cells with RU 28362 (10 nM), dexamethasone (10 nM) or aldosterone (100 nM), alone or in the presence of RU 38486 (100 nM). This latter steroid has relatively high affinity

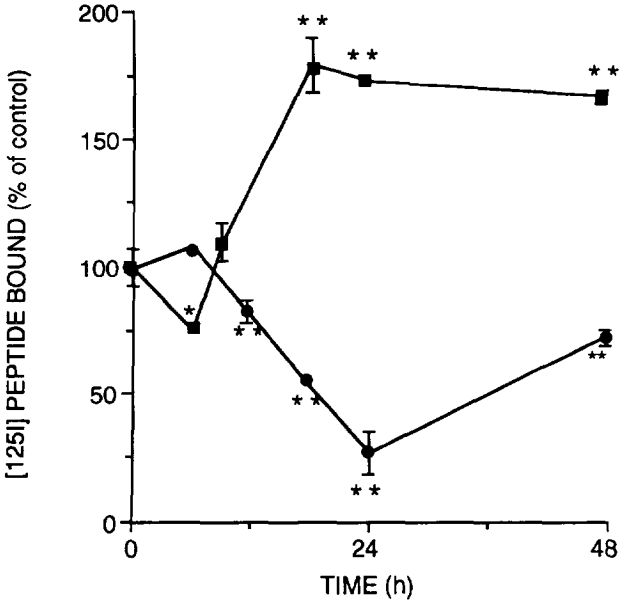

Fig. 1. Time-course of the effect of 100 nM RU 28362 on [^{125}I]endothelin-1 and [^{125}I]angiotensin II binding in VSMC. VSMC were grown to confluence in 10% FCS/DMEM, the medium changed to DMEM without FCS for 24 h, and then changed again to DMEM and steroid treatment begun. RU 28362 (20 μl of 5 μM in 0.5% ethanol) was added at time −48 h, −24 h, ... 0 h (ethanol alone) to wells and then the medium removed, the wells washed twice and [^{125}I]endothelin-1 (●) or [^{125}I]angiotensin II (■) binding studies performed as detailed in Methods. Non-specific binding was measured for each treatment by adding 1 μM of either endothelin-1 or angiotensin II and values so obtained subtracted from total binding; results shown are for specific binding. Data are expressed as % of control. Experiments were in triplicate and results shown are mean ± SEM of one representative experiment, of three separate experiments. *$P < 0.05$ vs control, **$P < 0.01$ vs control. Data were analysed by ANOVA.

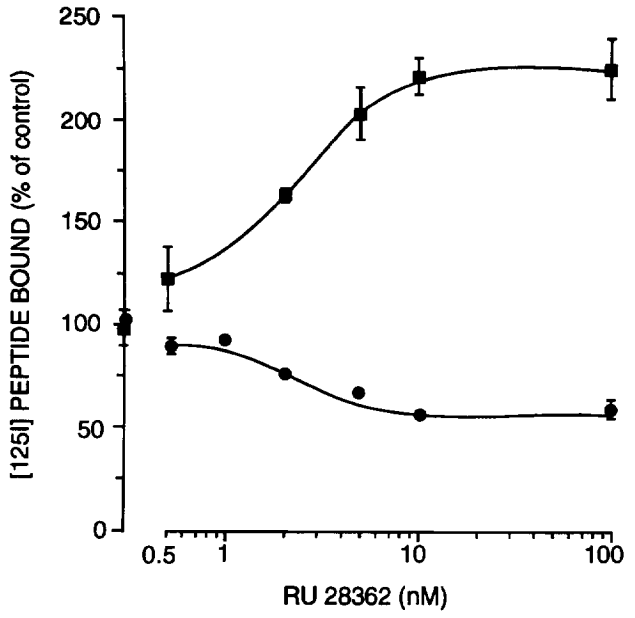

Fig. 2. Dose–response of the effect of RU 28362 on [^{125}I]endothelin-1 and [^{125}I]angiotensin II binding in VSMC. VSMC were grown to confluence in 10% FCS/DMEM, the medium changed to DMEM without FCS for 24 h, and then changed again to DMEM and steroid treatment begun. VSMC were treated for 24 h with 0, 0.2, 0.5, 2, 5, 10 or 100 nM RU 28362, the medium removed, the wells washed twice and [^{125}I]endothelin-1 (●) or [^{125}I]angiotensin II (■) binding studies performed as detailed in Methods. Non-specific binding was measured for each treatment by adding 1 μM of either endothelin-1 or angiotensin II and values subtracted from total binding; results shown are for specific binding. Data are expressed as % of control. Experiments were in triplicate and results shown are mean ± SEM of one representative experiment, of two separate experiments.

for both glucocorticoid and progesterone receptors in most species, and is an antagonist in both; it has negligible affinity for mineralocorticoid receptors.

As shown in Table 1, aldosterone lowers endothelin binding, and increases angiotensin II binding, when VSMC are exposed to the steroid in the absence of RU 38486. That this effect reflects aldosterone occupancy of classical glucocorticoid receptors is shown by the studies in which an equal concentration (100 nM) of RU 38486 is added to that of aldosterone. Under these conditions there is no difference in binding of either tracer to that seen in the presence of RU 38486 alone—i.e. there is no evidence for an action of aldosterone via high affinity, RU 38486-resistant mineralocorticoid receptors.

Competition studies performed to determine the receptor subtype affected by glucocorticoid treatment are shown in Fig. 4. [^{125}I]endothelin-1 binding was significantly decreased by addition of the ET_A receptor antagonist BQ123, and much less or not at all by the ET_B receptor agonist S6C in VSMC. Binding of [^{125}I]angiotensin II was significantly decreased by the AT-1 receptor antagonist DUP753 in VSMC, but not by the AT-2 receptor antagonist PD123319.

DISCUSSION

Although corticosteroids have long been known to affect vascular reactivity, the mechanism of their action on vascular responsiveness is not clear. *In vitro*, direct effects of both glucocorticoids and mineralocorticoids on vasculature have been reported. The data presented in this report clearly demonstrate that glucocorticoids modulate receptor levels for two major vasoactive agents in SHR VSMC in culture; endothelin-1 binding was decreased by RU 28362, a pure glucocorticoid agonist, and angiotensin II binding was increased. These effects are mediated via the Type II (glucocorticoid) receptor and are time- and dose-dependent. There appear to be no Type I (mineralocorticoid) receptor mediated effects of aldosterone on endothelin-1 or angiotensin II receptors, suggesting specificity of glucocorticoid action on vascular responsiveness.

There has been one previous report on the effects of glucocorticoids on AT-1 receptor levels in VSMC from WKY rats. In these studies glucocorticoids increased AT-1 mRNA levels [23], and the same authors have shown glucocorticoids to increase angiotensin II stimulated IP_3 formation in the same cells [9]. Taken together, these findings in WKY cells and our results in SHR are evidence for an increase in AT-1 levels by glucocorticoids, and could by analogy explain the increase in vascular reactivity observed *in vivo* in glucocorticoid treated humans [24]. Our data also show that levels of ET_A receptors are decreased by glucocorticoids in VSMC. This effect was time- and dose-dependent, mediated via Type II receptors and reflected a decrease in binding site number and not of affinity of the ligand for its receptor. A decrease in endothelin-1 binding in two VSMC lines (A7r5 and A-10 cells) has recently been reported [19, 25]. In one study no data on time-course, dose–response or level of inhibition were given, nor was it made clear whether the effect seen was on B_{max} or receptor affinity. In the other study Nambi *et al.* [18] showed similar results to those presented in this report in terms of inhibition and time of maximal inhibition, but a major discrepancy in terms of the dose–response curve. They reported an EC_{50} for the effect of dexamethasone of 20–30 pM, 100-fold lower than that reported in this paper for the effect of RU 28362 on endothelin-1 binding in VSMC; clearly a value of 2–3 nM is more in agreement with classical glucocorticoid dose–response curves. The decrease in endothelin-1 binding reported in all three studies is consistent with the 60% decrease in ET_A mRNA levels after 24 h treatment of A-10 VSMC with 10 nM dexamethasone [18]. Together these results represent clear evidence that glucocorticoids decrease ET_A levels in VSMC.

The time-course of the effect of glucocorticoids on endothelin-1 binding in our studies deserves particular attention. The effect of glucocorticoids is transient, despite continued exposure; whether or not this time-

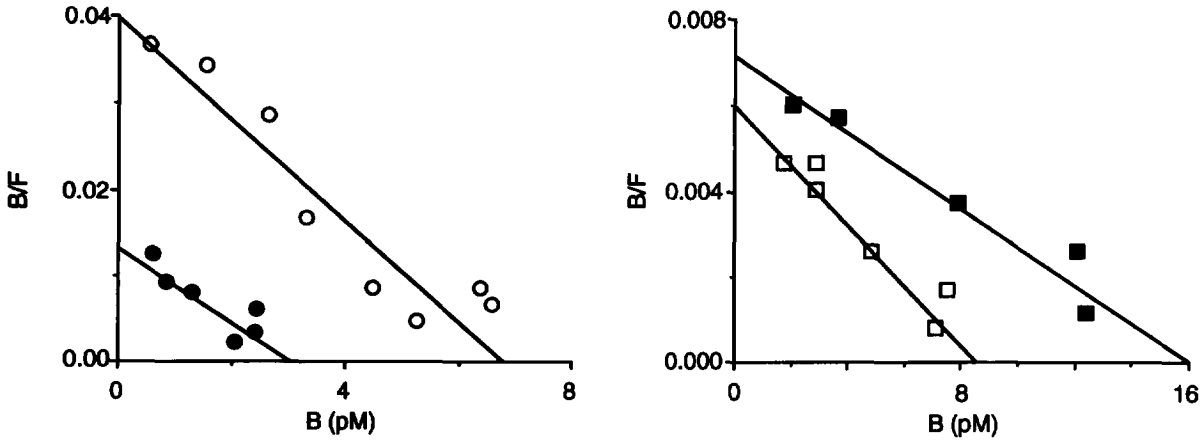

Fig. 3. Scatchard plot of the effect of RU 28362 on [^{125}I]endothelin-1 and [^{125}I]angiotensin II binding in VSMC. VSMC were grown to confluence in 10% FCS/DMEM, the medium changed to DMEM without FCS for 24 h, and then changed again to DMEM and steroid treatment begun. VSMC were treated for 24 h in the presence (closed symbols) or absence (open symbols) of 100 nM RU 28362, the medium removed, the wells washed twice and [^{125}I]endothelin-1 (left panel) or [^{125}I]angiotensin II (right panel) binding studies performed as detailed in Methods. Non-specific binding was measured for each treatment by adding 1 μM of either endothelin-1 or angiotensin II and the values subtracted from total binding. Experiments were in quadruplicate and results shown are those of a representative experiment of three (for endothelin-1 binding) and two (for angiotensin II binding) separate determinations. Data analysis was by LIGAND.

course is affected by the continued presence of endothelin-1 (as suggested by recent studies from Roubert *et al.* [26]) is currently being addressed, by studies on receptor mRNA levels in the presence or absence of the cognate ligands.

Whereas glucocorticoids are often considered pressor, in the present study they clearly have opposite effects on receptors for potent vasoconstrictor agents. While the increase in angiotensin II receptor levels is consistent with the increase in vascular responsiveness seen *in vivo* after glucocorticoid infusion [24], the role of a decrease in endothelin-1 binding suggests that the effect of glucocorticoids on vascular reactivity is not uniquely to increase vasoconstriction. One interpretation of this apparent dichotomy is that glucocorticoids may be acting to modulate as well as mediate the pressor response, particularly given the parallel time courses observed; glucocorticoid actions on the immune system, to both mediate and modulate the inflammatory response, are widely accepted [27]. Endothelin-1 is secreted by the endothelium and acts locally on VSM; it is the most potent vasoconstrictor

Table 1. Steroid effects on endothelin-1 and angiotensin II binding in vascular smooth muscle cells

	[^{125}I]ET-1 binding (% of control)		[^{125}I]A II binding (% of control)	
	Control	RU 38486 (100 nM)	Control	RU 38486 (100 nM)
Control	100 ± 1	97 ± 1	100 ± 11	112 ± 9
RU 28362 (10 nM)	73 ± 4*†	110 ± 2†‡§	172 ± 17*†	134 ± 2§
DEX (10 nM)	67 ± 1*†	114 ± 0.3*§‖	175 ± 5*†	138 ± 4§
Aldosterone (100 nM)	80 ± 5*†	91 ± 3	130 ± 5†	110 ± 11

VSMC were grown to confluence in 10% FCS/DMEM, the medium changed to DMEM without FCS for 24 h, the medium changed again to DMEM and steroid treatment begun. VSMC were treated for 24 h with either medium alone, 10 nM RU 28362, 10 nM dexamethasone, or 100 nM aldosterone in the presence or absence of 100 nM RU 38486. The medium was then removed, the wells washed twice and [^{125}I]endothelin-1 or [^{125}I]angiotensin II binding studies performed as detailed in Methods. Non-specific binding was measured for each treatment by adding 1 μM of either endothelin-1 or angiotensin II and the values subtracted from the total binding; results are expressed as % of specific binding compared to control. Data are expressed as % of control. Experiments were in triplicate and results shown are mean ± SEM values of one of two representative experiments.

*$P < 0.01$ vs control, †$P < 0.05$ vs control, ‡$P < 0.05$ vs RU 38486 alone, §$P < 0.01$ vs same treatment without RU 38486, ‖$P < 0.01$ vs RU 38486 alone.

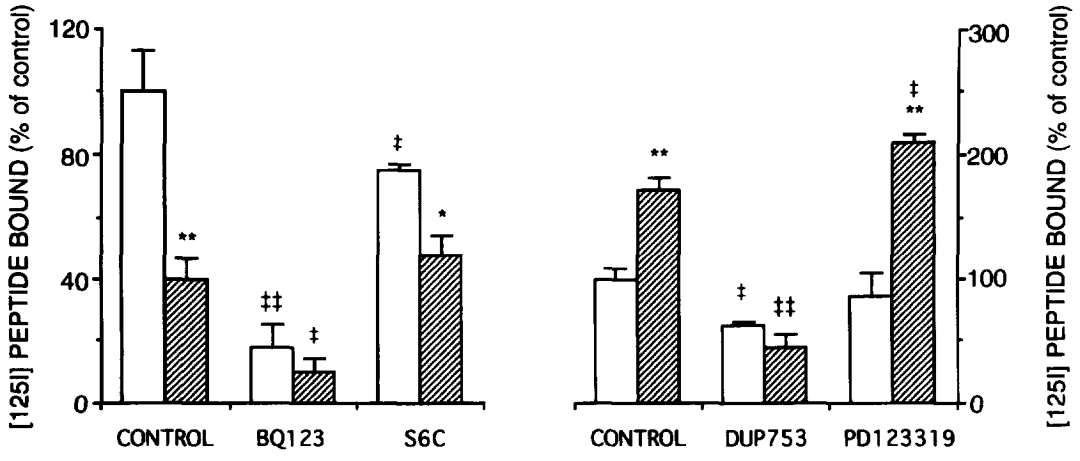

Fig. 4. [^{125}I]endothelin-1 and [^{125}I]angiotensin II binding in VSMC; characterization of receptor subtypes affected. VSMC were grown to confluence in 10% FCS/DMEM, the medium changed to DMEM without FCS for 24 h, and then changed again to DMEM and steroid treatment begun. VSMC were treated for 24 h with media alone (open bars) or 100 nM RU 28362 (hatched bars), the medium removed, the wells washed twice and binding studies performed as detailed in Methods. VSMC were incubated with: [^{125}I]endothelin-1 (left panel) in the presence or absence of 10 nM of BQ123 or S6C; [^{125}I]angiotensin II (right panel) in the presence or absence of 100 nM of DUP753 or PD123319. Non-specific binding was measured for each treatment by adding 1 μM of either endothelin-1 or angiotensin II and values subtracted from total binding; results shown are for specific binding. Data are expressed as % of control and are mean ± SEM values from one experiment done in quadruplicate. ‡‡$P < 0.01$ vs control, ‡$P < 0.05$ vs control, **$P < 0.01$ vs same treatment without RU 28362, *$P < 0.05$ vs same treatment without RU 28362. Data were analysed by ANOVA.

known, its action is long lasting [19] and has also been postulated to have proliferative effects [28]. Its synthesis and secretion is stimulated by pro-inflammatory agents in the endothelium [19, 29] and therefore it has been implicated in the inflammatory response, given its localization at the site of lesion [19]. Glucocorticoids have also been proposed to be implicated in the inflammatory response in the vasculature by their effects on prostaglandin release [16]. Therefore, the decrease in endothelin-1 binding observed in this study by glucocorticoid treatment of VSMC may suggest a role for glucocorticoids in the modulation of the vascular response to inflammation, rather than a primary role as a simple vasoconstrictor.

The presence of Type I (mineralocorticoid) receptors has been clearly established in the vasculature by the demonstration *in vitro* of high affinity mineralocorticoid binding in aortic cytosol from rats [30] and rabbits [31]. *In vivo* studies [32] have also clearly demonstrated both Type I receptor binding and 11β-hydroxysteroid dehydrogenase activity in mesenteric vascular arcade. In this study we have shown Type II mediated effects of glucocorticoids and aldosterone on receptors for vasoactive peptides in VSMC, with no Type I mediated effect able to be demonstrated. There are conflicting findings in the literature on mineralocorticoid effects in the vasculature, both *in vivo* and *in vitro* [3]. Jazayeri and Meyer [21] reported an increase in β-adrenergic receptors after treatment of VSMC with either aldosterone or spironolactone, and Ullian *et al.* reported aldosterone–angiotensin II interactions on protein synthesis [33]. On the other hand, no specific mineralocorticoid domain of action in VSMC cultures or cardiac muscle cells could be demonstrated on two dimension gel electrophoresis [5]. More recently, Kornel *et al.* [34] have provided convincing evidence for RU 38486-resistant effects of aldosterone on VSMC Ca^{2+} flux *in vitro*, effects interestingly seen after 7–10 days of steroid exposure, but not after only 1–2 days, comparable to the time of exposure used in the present studies.

In conclusion, we have demonstrated that glucocorticoids modulate receptor levels for two major constrictor agents in SHR VSMC. The effects were on receptor number and were in opposite directions, suggesting a complex physiological regulation of vascular responsiveness by glucocorticoids. In contrast, occupancy of Type I (mineralocorticoid) receptors appears to have no effect on either the levels of angiotensin II or endothelin-1 receptors.

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