Glucocorticoid-Related Signaling Effects in Vascular Smooth Muscle Cells

Gergö A. Molnar, Carsten Lindschau, Galyna Dubrovskva, Peter R. Mertens, Torsten Kirsch, Marcus Quinkler, Maik Gollasch, Stefanie Wresche, Friedrich C. Luft, Dominik N. Muller, Anette Fiebeler

**Abstract**—Mineralocorticoid receptor blockade protects from angiotensin II–induced target-organ damage. 11β-Hydroxysteroid dehydrogenase type 2 protects the mineralocorticoid receptor from activation by glucocorticoids; however, high glucocorticoid concentrations and absent 11β-hydroxysteroid dehydrogenase type 2 in some tissues make glucocorticoids highly relevant mineralocorticoid receptor ligands. We investigated the effects of corticosterone (10^{-6} to 10^{-12} mol/L) on early vascular mineralocorticoid receptor signaling by Western blotting, confocal microscopy, and myography. Corticosterone initiated extracellular signal–regulated kinase 1/2 phosphorylation in rat vascular smooth muscle cells at ≥10^{-11} mol/L doses. Protein synthesis inhibitors had no effect, indicating a nongenomic action. Corticosterone also stimulated c-Jun N-terminal kinase, p38, Src, and Akt phosphorylation at 15 minutes and enhanced angiotensin II–induced signaling at 5 minutes. A specific epidermal growth factor receptor blocker, AG1478, as well as the Src inhibitor PP2, markedly reduced corticosterone-induced extracellular signal–regulated kinase 1/2 phosphorylation, as did preincubation of cells with the mineralocorticoid receptor antagonist spironolactone. Silencing mineralocorticoid receptor with small interfering RNA abolished corticosterone-induced effects. Corticosterone (10^{-9} mol/L) enhanced phenylephrine-induced contraction of intact aortic rings. These effects were dependent on the intact endothelium, mineralocorticoid receptor, and mitogen-activated protein kinase kinase 1/extracellular signal-regulated kinase signaling. We conclude that corticosterone induces rapid mineralocorticoid receptor signaling in vascular smooth muscle cells that involves mitogen-activated protein kinase/extracellular signal–regulated kinase–dependent pathways. These new mineralocorticoid receptor–dependent signaling pathways suggest that glucocorticoids may contribute to vascular disease via mineralocorticoid receptor signaling, independent of circulating aldosterone.

**Key Words:** corticosterone ■ angiotensin ■ phenylephrine ■ mineralocorticoid receptor ■ epidermal growth factor receptor

**Clinical** trials have shown that mineralocorticoid receptor (MR) blockers reduce morbidity and mortality and protect against progressive renal disease. Although aldosterone has been seen as the culprit, glucocorticoids bind to the MR with the same affinity at physiological concentrations and exert similar effects on various targets, including vessels. These observations are consistent with the fact that Cushing’s syndrome patients have a high cardiovascular mortality. The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) converts MR-active cortisol to cortisone, which does not bind to the MR. 11β-HSD2 inactivity occurs in the syndrome of apparent mineralocorticoid excess or in licorice abuse and allows cortisol to activate the MR. Affected persons show enhanced vasoconstrictor dermal vessel responses to cortisol and symptoms mimicking locally high aldosterone levels. Because cortisol levels are >1000-fold higher than aldosterone levels, cortisol is an MR ligand even under physiological conditions, particularly in tissues not expressing 11β-HSD2. We tested the hypothesis that corticosterone, the physiological glucocorticoid in rats and mice, may exert aldosterone-like effects on early signaling in the vasculature and that these effects are mediated via MR and mitogen-activated protein kinase kinase (MEK)/extracellular signal–regulated kinase–dependent pathways. Because rapid and protein synthesis-independent aldosterone signaling has been described previously, we reasoned that...
corticosterone might also signal along these lines. We also examined corticosterone responses and signaling in aortic rings.

**Materials and Methods**

**Cell Culture**

Aortic vascular smooth muscle cells (vascular smooth muscle cells [VSMCs]) were isolated from Sprague-Dawley rats as described previously. We followed all of the requirements of the American Physiological Society, and local authorities approved the studies. We used passages 3 to 8 after phenotyping by staining VSMCs for muscle- 
globin. Cells were treated with corticosterone, angiotensin (Ang II; both Sigma), or aldosterone (Cinalfa). The following blockers were used as indicated: AG 1478, LY294002, PD98059, PP2, actinomycin, cycloheximide (all Calbiochem), spironolactone, RU 486, and carbenoxolone (CBX; all Sigma). All of the experiments were performed under 24-hour serum- 
free conditions. For small interfering RNA (siRNA) experiments, cells were transfected with specific siRNA (Dharmacon) directed against MR (Sigma). All of the experiments were performed under 24-hour serum- 
free conditions with EGF, Ang II, and Ang III (both Sigma). All of the experiments were performed under 24-hour serum- 
free conditions.

**Immunohistochemistry**

Confocal microscopy was performed as described previously. At least 50 to 80 cells from ≥3 experiments were examined at each treatment point by 2 blinded investigators. Quantification was by histogram functions using the MRC laser sharp software. The subcellular regions were outlined manually, and mean fluorescence intensities were obtained for the regions delineated with data presented as mean fluorescence intensity in each cell area. Rabbit and mouse antiphospho-ERK1/2 antibodies (Cell Signaling; 1:200) or phospho-Src (Cell Signaling; 1:100) was used in the studies.

**Western Blot**

The following primary antibodies were used: polyclonal ERK1/2 (Cell Signaling; 1:1000), phospho-ERK1/2 (Cell Signaling; 1:1000), phospho-c-Jun N-terminal kinase (Cell Signaling; 1:1000), phospho-epidermal growth factor receptor (EGFR; Cell Signaling; 1:1000), phospho-Akt (Cell Signaling; 1:500), phospho-Src (Cell Signaling; 1:1000), phospho-ERK1/2 (Cell Signaling; 1:1000), phospho-ERK1/2 (Cell Signaling; 1:500), and mouse antiphospho-ERK1/2 antibodies (Cell Signaling; 1:200) was used in the studies.

**Aortic Ring Contraction**

All of the animal procedures were in accordance with institutional guidelines corresponding with those of the American Physiological Society. Male Sprague-Dawley rats (250 to 300 g, Charles River, Berlin, Germany) were killed, and the thoracic aorta removed, quickly transferred to cold (4 °C) oxygenated (95% O2/5% CO2) physiological salt solution, and dissected into 5-mm rings as described previously, with perivascular fat and connective tissue removed. After 1 hour of equilibration, aortic ring contractile force was measured isometrically by standard bath procedures as described previously. The composition of physiological salt solution (in mmol/L) was 119 NaCl, 4.7 KCl, 1.2 KHPO4, 25 NaHCO3, 1.2 MgSO4, 11.1 glucose, and 1.6 CaCl2, and the volume of the bath solution was 20 mL. Cumulative dose-response curves were obtained for phenylephrine in the absence or presence of corticosterone or spironolactone, with tension expressed as a percentage of the steady-state tension (100%) found in isotonic external KCl at 60 mmol/L. In some rings, the endothelial layer was disrupted by gently rubbing the luminal surface with a forceps’ tip. Endothelium was accepted as “nonfunctional” if no relaxation was seen with 10 μmol/L of acetylcholine.

**Statistics**

Data were analyzed by SPSS 13.0 and those with a normal distribution (Kolmogorov-Smirnov test) are shown as means±SEMs. Statistical significance was tested by unpaired t test, or, in case of multiple groups, ANOVA with posthoc correction according to Bonferroni. A value of P<0.05 was considered statistically significant.

**Results**

**Corticosterone Stimulates ERK1/2 Phosphorylation in VSMCs**

The high supraphysiological dose of corticosterone (10−8 mol/L) used in the initial studies on ERK1/2 phosphorylation showed that ERK1/2 phosphorylation was maximal at 15 minutes (Figure 1A). In the steroid classification system, corticosterone has been called “Kendall’s” compound B. We used “B” to designate corticosterone in the figure. At 10−8 mol/L, corticosterone induced ERK1/2 phosphorylation that peaked within 10 minutes; similar results were found by immunohistochemistry and confocal microscopy (n=4: control: 1.0; B: 10 minutes: 3.25±1.03). Lower and physiologically relevant free corticoste- rone doses (10−9 to 10−11 mol/L) induced ERK1/2 signaling at 15 minutes (supplement Figure S1, available online at http://hyper.ahajournals.org). When the corticosterone concentration was reduced to 10−11 mol/L in a second series of dose-response studies, we observed diminished ERK1/2 phosphorylation (Figure 1B). We also investigated corticosterone-induced ERK1/2 phosphorylation in the presence of the transcription and protein synthesis inhibitors actinomycin D (preincubation: 30 minutes; 1 μg/mL; n=3 to 5: control: 0.75±0.25; B: 3.03±0.49, actino- mycin D: 2.56±0.32, actinomycin D+corticosterone: 3.98±0.22) and cycloheximide (preincubation: 30 minutes; 5 μg/mL; n=3 to 5: control: 1.2±0.38, corticosterone: 3.22±0.55, cycloheximide: 3.41±0.12, cycloheximide+corticosterone, 4.06±0.17). Neither inhibitor affected short-term ERK1/2 phos- phorylation, supporting a nongenomic effect of corticosterone.

**Corticosterone Stimulates c-Jun N-Terminal Kinase, Akt, and Src Phosphorylation in VSMCs**

We next investigated downstream corticosterone-stimulated rapid pathways. Stimulation of VSMCs with corticosterone (10−7 mol/L) for 15 minutes induced c-Jun N-terminal kinase phosphorylation (Figure S2A), p38 mitogen-activated protein kinase phosphorylation (Figure S2B), and Src phosphorylation (Figure S2C), with eIF-4e used as a loading control.
Corticosterone-induced Src phosphorylation was completely inhibited when cells were preincubated with the tyrosine kinase inhibitor PP2 (10⁻⁵ mol/L) for 30 minutes (Figure S2D). In addition, corticosterone induced Akt phosphorylation at 15 minutes (Figure 2A), which was completely abolished by preincubating the cells with the phosphatidylinositol 3-kinase inhibitor LY294002 (10⁻⁵ mol/L) for 30 minutes. LY294002 did not affect corticosterone-induced ERK1/2 phosphorylation (Figure 2B).

**11β-HSD2 Activity Does not Affect Corticosterone Levels**

We determined the 11β-HSD2 activity under our experimental conditions. The percentage of substrate metabolized from corticosterone to 11-dehydrocorticosterone was 1% in each experiment. Furthermore, we used the 11β-HSD2 inhibitor CBX (preincubation for 30 minutes). CBX at a dose of 10⁻⁹ mol/L did not affect corticosterone-induced ERK1/2 phosphorylation (n=12: control: 4.71±0.18, corticosterone: 9.10±0.69, CBX: 4.48±0.16, CBX+B: 8.77±0.29). Thus, 11β-HSD2 was not active under the conditions of our experiments.

**Corticosterone-Induced Signaling Is Mediated Through MR**

We preincubated VSMCs for 30 minutes with spironolactone (10⁻⁷ mol/L). We chose this low dose according to experiences of other groups, that observed partial agonist effects of spironolactone at higher doses on smooth muscle contraction. We suggest that equimolar spironolactone doses blocked ERK phosphorylation and that these doses may be related to our experimental setting with a 30-minute preincubation with spironolactone given before stimulation. We then exposed the cells to corticosterone (10⁻⁷ mol/L) for 15 minutes. Spironolactone prevented corticosterone-induced ERK1/2 phosphorylation at 15 minutes, as shown by immunoblotting (Figure S3A) and by confocal microscopy (Figure S3B). Similar results were observed with the more specific MR blocker eplerenone at 10⁻⁵ mol/L (Figure S3C). To confirm the findings, we used siRNA targeting the MR. Successful silencing of the MR was shown by quantitative RT-PCR measuring mRNA expression of the MR, which was reduced by 80% in the MR siRNA-transfected cells compared with cells transfected with nontargeting control RNA (Figure S4). siRNA against the MR prevented corticosterone, aldosterone, and Ang II–induced ERK1/2 phosphorylation but did not affect the response to phorbol ester. In VSMCs transfected with nontargeting control siRNA, aldosterone, Ang
II–induced effects, we stimulated VSMCs with Ang II (10⁻⁶ mol/L) for 30 minutes (Figure 5B). Preincubation of the rings with spironolactone (10⁻⁶ mol/L) for 30 minutes partially reduced the corticosterone-dependent effects (Figure 5C) but did not affect phenylephrine-dependent contractions in the absence of corticosterone. Similarly, preincubation with the GR blocker RU486 (10⁻⁶ mol/L) reduced the corticosterone-dependent effects (200 nM phenylephrine plus corticosterone without RU486 92±3% versus with RU486 75±6%, although, RU486 did not enhance suppressive effects of spironolactone.

To test the role of MEK1/ERK1/2 on corticosterone-induced enhancement of phenylephrine-dependent contraction, we preincubated rings with the specific MEK1 inhibitor PD98059 (10⁻⁵ mol/L, 30 minutes). PD98059 reduced phenylephrine-related effects on phenylephrine-dependent vasoconstriction but did not inhibit the phenylephrine-dependent contraction in the absence of corticosterone (Figure 5D).

**Discussion**

Our data suggest that corticosterone exerts rapid effects on signaling in VSMCs in a fashion similar to aldosterone. Corticosterone-induced effects involve signaling pathways that include phosphorylation of mitogen-activated protein kinases, Src, and Akt. ERK1/2–induced signaling depended on an intact MR and a functional EGFR. Corticosterone-induced effects on aortic ring contraction were opposite to aldosterone-induced effects, at least ex vivo. Although aldosterone diminishes phenylephrine-induced contraction, corticosterone enhanced phenylephrine-induced aortic ring contraction, an effect dependent on an intact endothelium and partially reduced by MR blockade or an MEK1–ERK1/2 blocker.

The MR and GR both belong to the same nuclear hormone receptor superfamily, and they share high sequence identity. Both receptors bind glucocorticoids, cortisol in humans, and corticosterone in rats and mice with high affinity. However, aldosterone binds to the MR with high affinity, whereas its affinity for the GR is much lower. Because aldosterone plasma concentrations are 3 orders of magnitude lower than cortisol or corticosterone concentrations, cortisol should occupy most of the MR. This excess is diminished 10-fold by the stronger plasma protein binding of cortisol by a corticosteroid-binding globulin (3% unbound cortisol) compared with the weak protein binding of aldosterone by albumin (30% unbound aldosterone).

In transfection studies, cortisol showed a 10-fold weaker transactivation activity of the MR compared with aldosterone despite an equal binding affinity. In addition, the cortisol-MR receptor complex seems to be less stable than the aldosterone-MR receptor complex because of a different receptor conformation. This state of affairs leads to the dissociation of cortisol from the MR that is 2 to 4 times faster than that of aldosterone. Furthermore, the exact mechanism regarding how cortisol and aldosterone enter the cell is not clear, and there could be further differences between these steroids because of the 11-18 hemiketal group of aldosterone. However, to allow exclusive aldosterone activation of MR in epithelial cells, the enzyme 11β-HSD2 is expressed and metabolizes cortisol and corticosterone to their inactive metabolites cortisone and 11-dehydrocorticosterone. In nonepithelial cells, which express...
MR but not 11β-HSD2, the MR is probably occupied by glucocorticoids. To complicate matters further, several studies have shown that cortisol can block the actions of aldosterone, suggesting that, in many circumstances, cortisol occupies the MR and acts as an MR antagonist.\textsuperscript{21,22} To what extent further regulatory mechanism, such as coactivator complexes and conformational interactions of MR domains, influence the ligand specificity, transactivation, or repression at the MR, as well as the exact ligand concentration at the intracellular steroid receptor, is unknown.\textsuperscript{23,24}

Our data indicate that corticosterone activates MR in VSMCs to trigger the phosphorylation of proteins that are central to cell proliferation and differentiation. We believe that these effects are mediated through the classical MR, because MR silencing, in addition to the MR antagonists spironolactone and eplerenone, prevented corticosterone signaling. We interpret these data as indicating that corticosterone-induced effects could mimic aldosterone-induced events. These findings may extend our understanding of the MR and how the receptor mediates unexplained pathways. They raise the possibility that glucocorticoids activate MR signaling in nonepithelial tissue and could thereby increase cardiovascular risk. Moreover, the data could have implications in terms of clinical MR blockade with currently available drugs.

The rapid effects of corticosterone on protein phosphorylation are in line with results of Gros et al.,\textsuperscript{14} who showed that cortisol stimulated myosin light-chain phosphorylation in VSMCs within 30 minutes and that this effect was blocked by spironolactone. Corticosterone can be an agonist for both MRs.
Corticosterone (B) potentiates aortic ring contraction. A, B (10^{-9} mol/L; n=9)-enhanced phenylephrine (Phe; 50 to 200 nmol/L; n=6) induced contraction (given in percentage of 60 mmol/L of KCl-induced contraction; *P<0.05; 50 nmol/L of B and Phe were given simultaneously; afterward, increasing doses of Phe were added to the bath solution, and the total stimulation time was 10 minutes). B, Mechanical removal of the endothelium before the experiments abolished the observe effect of B (10^{-9} mol/L; n=6). C, Spironolactone (Spi; 10^{-5} mol/L; n=12; 30 minutes of preincubation before stimulation) reduced B-induced enhancement of Phe contractions; *P<0.05. C, PD98059 (PD; 10^{-5} mol/L; n=6 each; 30 minutes of preincubation before stimulation) reduced B-induced enhancement of Phe-dependent contractions. PD98059 did not affect Phe-induced contractions in the absence of B (*P<0.05).

**Figure 5.**

and for GRs, and both receptor classes are expressed in VSMCs. They also have a common evolutionary precursor, display high sequence homology, and their DNA binding domains bind to the same regulatory elements. GRs mediate anti-inflammatory and immunosuppressive signals, and in non-epithelial tissue MR activation produces the opposite effects. To test whether the GR plays a role in the rapid effects, we showed that RU486 did not inhibit corticosterone-induced signaling in VSMCs. However, RU486 partially inhibited corticosterone-induced effects on aortic ring contraction, suggesting that in vivo neighboring cells and coexisting factors may modulate the response to corticosterone. In vivo, 11β-HSD2 is expressed in VSMCs and converts to cortisol and to receptor-inactive 11-keto products. Alzamora et al demonstrated that the 11β-HSD2 inhibitor, CBX, significantly enhanced cortisol-induced pH changes at doses as low as 0.5 nmol/L in endothelium-denuded aortic rings. Their findings demonstrated the principle of cortisol-relevant effects on the vasculature. Nonetheless, whether and how 11β-HSD2 influences composition or stability of steroid-MR complexes with associated coactivators or repressors to modulate MR activity chronically in vivo remain to be investigated.

Sato et al and Ullian et al observed that corticosterone upregulates Ang II type 1 receptor mRNA and protein levels in cultured VSMCs. However, these effects cannot explain the enhancing responses that we observed for corticosterone on Ang II–induced effects after 5 minutes. We suggest that ligand binding to the MR initiates shifting and redistribution of different receptors forming a protein complex, thereby facilitating transmission of an activating signal. We have shown previously that the Ang II type 1 receptor and the MR form a functional protein complex and that MR blockade reduces Ang II–induced ERK1/2 phosphorylation. When we stimulated VSMCs after MR silencing with Ang II, ERK1/2 phosphorylation was markedly reduced in siRNA-transfected cells but was unaffected in control-transfected cells. We conclude that corticosterone can activate MR producing rapid signaling and, similar to aldosterone, enhances Ang II–induced signaling via MR. A third likely candidate member of the receptor complex is EGFR in that AG1478, which specifically inhibits EGFR kinase activity, prevents corticosterone-induced signaling. We suggest that additional stimuli at other receptors involved in the complex may also influence MR activity.

**Perspectives**

Our data have clinical implications. Hoen et al reported recently that cortisol administered to patients with traumatic shock increases the sensitivity to α1-adrenoceptor stimulation independent of adrenal reserve, thus supporting the view that glucocorticoids modulate vascular function and tone. Tauchmanova et al showed that patients with symptoms of even a
minor degree of Cushing’s syndrome have increased cardiovascular risk. Güder et al.14 observed that high cortisol levels in heart failure patients were independent predictors of increased mortality risk. Total cortisol and its metabolite concentrations are higher in hypertensive persons with glucose intolerance than in normotensive control subjects.15 Our data could contribute to the understanding of the role of MR in the progression of cardiovascular disease.

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Disclosures

None.

References

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Glucocorticoid-related signaling effects in vascular smooth muscle cells

Short title: Glucocorticoids and vessels

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**Figure S1.** Corticosterone (B) stimulates ERK 1/2-phosphorylation in VSMC. Dose dependent effects of B on ERK 1/2-phosphorylation (10^{-7} mol/l to 10^{-12} mol/l; n=2; *p<0.05)

**Figure S2.** Corticosterone (B) stimulates JNK-, p-38- and Src-phosphorylation in VSMC. A. B (10^{-7} mol/l) stimulated JNK-phosphorylation at 15 min (n=5; *p<0.05). B. B (10^{-7} mol/l) stimulated p38-phosphorylation at 15 min (n=6; *p<0.05). C. B (10^{-7} mol/l) stimulated Src-phosphorylation at 15 min (n=6; *p<0.05). eIF-4e-protein was used as loading control. D. (B 10^{-7} mol/l induced SRK 1/2-phosphorylation at 15 min can be blocked with PP2 (10^{-5} mol/l), a specific Src-inhibitor (PP2 pre-incubation for 30 min).

**Figure S3.** Corticosterone (B) signaling is mediated through the mineralocorticoid receptor (MR). A. Spironolactone (Spi; 10^{-7} mol/l; pre-incubation for 30 min) reduced B-induced ERK 1/2-phosphorylation at 15 min (n=5; *p<0.05 vs. control, **p<0.05 vs. B). B. The same effects were observed with immunofluorescence with confocal microscopy. C. The selective MR-blocker Eplerenone (EPL; 10^{-5} mol/l; pre-incubation for 30 min) reduced B and Ald- induced ERK 1/2-phosphorylation at 15 min; evaluation with immunofluorescence and confocal microscopy.

**Figure S4.** siRNA targeting the mineralocorticoid receptor (MR) reduces MR-protein expression in vascular smooth muscle cells. VSMC transfected with non-targeting siRNA served as control (picture on the left), effects of MR-targeting siRNA are shown on the right. Immunohistochemistry was performed 3 days after transfection.

**Figure S5.** GR does not transmit corticosterone induced ERK1/2 phosphorylation in VSMC. RU486 (10^{-6} mol/l; pre-incubation for 30 min) has no effect on B-induced ERK 1/2-phosphorylation at 15 min (n=2).
Figure S2

A

pJNK

elF-4e

Ctr    B

arbitrary units

Ctr    B

*  

B

p38

elF-4e

Ctr    B

arbitrary units

Ctr    B

*  

C

pSrc

elF-4e

Ctr    B

arbitrary units

Ctr    B

*  

D

pSrc

Ctr    PP2

PP2 + B
Figure S3

A

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B

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C

pERK 1/2
Figure S4

MR-Expression

Non-targeting siRNA  MR-targeting siRNA
Figure S5

p-ERK 1/2

eIF-4e

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