Rapid effects of aldosterone on clonal human vascular smooth muscle cells

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Rapid effects of aldosterone on clonal human vascular smooth muscle cells. Am J Physiol Cell Physiol 292: C788–C794, 2007. First published September 13, 2006; doi:10.1152/ajpcell.00407.2006.—It has been increasingly appreciated that aldosterone elicits acute vascular effects through nongenomic signaling pathways. Our previous studies demonstrated that aldosterone attenuated phenylephrine-mediated constriction in intact vessels [via phosphatidylinositol 3-kinase-dependent nitric oxide synthase activation] but enhanced vasoconstrictor responses in endothelium-denuded arteries. To determine the mechanism of this vasoconstrictor response, we assessed the effect of aldosterone on myosin light-chain phosphorylation and contraction in clonal adult human vascular smooth muscle cells. Acute aldosterone exposure mediated dose-dependent myosin light-chain phosphorylation, inhibited by spironolactone and phosphatidylinositol 3-kinase inhibition. These rapid effects of aldosterone were mimicked by estradiol and hydrocortisone and were also inhibitable by both spironolactone and eplerenone. In parallel to its effects on myosin light-chain phosphorylation, aldosterone mediated dose-dependent contraction responses that were inhibited by spironolactone. Comparable contractile responses were seen with both 17β-estradiol and hydrocortisone. In total, these data are consistent with a mechanism of acute aldosterone-mediated contraction common to both glucocorticoids and estrogen. Steroid-mediated vasoconstriction may represent an important pathobiological mechanism of vascular disease, especially in the setting of preexisting endothelial dysfunction.

* Aldosterone and other vasoactive steroids are being increasingly appreciated as important physiological and pathophysiological regulators of cardiovascular function. The traditional view of the cardiovascular actions of aldosterone has focused on regulation of electrolyte homeostasis and extracellular fluid volume via the promotion of sodium retention and potassium excretion in the renal collecting duct (and via transcriptional regulation). However, there is mounting evidence for aldosterone-mediated responses beyond the collecting duct (4), specifically aldosterone effects in cardiac, vascular, and endothelial cells (16). These include effects on cardiac hypertrophy (7) and cardiac and vascular fibrosis (3, 8, 37). Furthermore, these effects have been suggested to be important both physiologically and pathologically (11). These extrarenal effects of aldosterone have been suggested to contribute to the deleterious effects of aldosterone, indicated by the “positive” effects of aldosterone receptor antagonists in patients with congestive heart failure or after myocardial infarction (25, 26).

The molecular mechanism(s) by which aldosterone mediates its extrarenal effects is unclear. Aldosterone’s role in renal salt and water balance generally has been ascribed to transcriptional regulation. However, there has been increasing evidence of the rapid (so-called nongenomic) actions of aldosterone (9), including acute vascular effects.

The rapid vascular effects of aldosterone reflect a balance between 1) endothelial and vascular smooth muscle actions and 2) vasodilator and vasoconstrictor mechanisms, much like those of several (G protein-coupled receptor) agonists, such as acetylcholine, catecholamines, and endothelin that act on multiple targets in blood vessels.

Work from our laboratory (and those of others) has described rapid vascular effects of aldosterone via both endothelium-dependent and endothelium-independent mechanisms. Our group (20) had previously characterized the endothelial mechanism of aldosterone that functionally results in attenuation of vasoconstriction. This effect is predominantly due to nitric oxide synthase (NOS) activation acting via a phosphatidylinositol 3-kinase (PI3-kinase)-dependent pathway. These endothelial-dependent aldosterone effects were inhibited by the classical mineralocorticoid receptor (MR) antagonist spironolactone (20). However, in endothelium-denuded preparations, aldosterone mediates constriction, an effect functionally antagonistic to that observed when there is an intact endothelial cell layer (20). The mechanism underlying this vasoconstrictor response was not elucidated in our initial studies.

Dual, functionally antagonistic effects of aldosterone have also been described in other animal models, as well as in humans. These divergent effects probably account for the initial “contradictory” reports of both vasodilator and vasoconstrictor aldosterone effects (1, 10, 27, 29–31, 39). In retrospect, these reports highlight the more recent appreciation that aldosterone mediates endothelium-dependent vasodilation that is “buffered” by a direct vasoconstrictor effect on vascular smooth muscle (or vice versa). The net expression of these actions appears to be dependent on the species, on the specific vascular bed investigated, and on endothelial integrity.

The mechanism underlying aldosterone’s endothelium-independent vasoconstrictor effect is unclear but may be especially important in settings of endothelial dysfunction, as in hypertension and atherosclerotic disease. On the basis of these uncertainties, we studied the acute vasoconstrictor effects of aldosterone (as well as estrogen and the glucocorticoid hydrocortisone) in isolated human cultured vascular smooth muscle cells. Data to be presented demonstrate that aldosterone mediates functional contractile responses paralleling myosin light-chain phosphorylation. Moreover, other vasoactive steroids mimic this effect.

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MATERIALS AND METHODS

Cell culture. Experiments were performed with the maturation-
competent human vascular smooth muscle cell line HITC6, generated
from the human internal thoracic artery, as previously described (18, 19). Cells were maintained in culture in M199 media with 10% FBS
with 1.8 mM calcium, at a pH of 7.2. HITC6 cells have been
previously characterized in detail with respect to their ability to
contract in response to vasoactive compounds (19).

Analysis of 20-kDa myosin light-chain phosphorylation. Phosphor-
ylation of the 20-kDa regulatory myosin light chain (MLC20) was
measured by previously published methods (24), with modifications
as noted below. Cells were treated with aldosterone, hydrocortisone,
estriol, or phenylephrine for various times in M199 media (with
0.1% BSA), as above, at 37°C after 6 days of serum deprivation. After
treatment, cells were washed twice with ice-cold PBS, and cellular
proteins were precipitated in ice-cold 10% TCA containing 2 mM
DTT. The resulting precipitates were washed in acetone containing
2 mM DTT and air dried for 30 min at room temperature. The dry pellet
was then dissolved in 70 µl of sample containing 8 M urea, 20
mM Tris, 23 mM glycine, and 10 mM DTT, pH 8.6. The separation
of unphosphorylated and phosphorylated forms of MLC20 was ac-
complished with urea-glycerol PAGE followed by immunoblot anal-
ysis. Urea-solubilized samples (50 µl) were separated on a 10%
glycerol-PAGE at 400 V for 1.5 h. Separated proteins were then
electroblotted to polyvinylidene difluoride membrane and detected by
immunoblotting using anti-MLC20 antibody (Santa Cruz Biotech).

Assessment of smooth muscle cell contraction. Smooth muscle cells
were plated at a density of ~4,000 cells/cm² 24 h before experimen-
tation. Plates were placed in a temperature-controlled chamber main-
tained at 37°C (Bionomic controller, 20/20 Technology) on an in-
verted microscope (Axiovert S100, Zeiss). Plates were equilibrated
for 15 min on stage before the start of 25-min baseline recordings. In
those cells assessed to demonstrate 1) baseline stability and 2) con-
trollable potential, the progression of smooth muscle cell contraction
was evaluated by time-lapse video microscopy with a digital recording
system. Cells were analyzed for their responses to steroids and/or
inhibitors if 1) their total cell area did not change more than 5% over
the last 5 min of the 25-min baseline recording period and 2) the
subsequent addition of ionomycin (1 µM) or phenylephrine (10 µM)
mediated a ≥25% reduction in cell area. Images were obtained every
30 s, and the extent of contraction was assessed by determining the
change in cell area by tracing the individual cells using image analysis
software (ImagePro 3.1). Contraction data were expressed as a per-
centage of the ionomycin-mediated response.

Statistical analysis. For two-sample comparisons, the statistical
significance of differences was determined by unpaired Student’s
t-test. For multiple group comparisons, initial analysis by one-way
ANOVA was followed by Dunnett’s multiple comparison tests. * P <
0.05 on a two-sided test was taken as a minimum level of significance.

Materials. Phenylephrine (10 µM), ionomycin (1 µM), aldosterone
(0.1–100 nM), LY-294002 (50 µM), LY-303511 (50 µM), spirono-
lactone (0.1–100 µM), estradiol (0.1–100 nM), and hydrocortisone
(0.1–100 nM) were purchased from Sigma. Eplerenone (10 µM) was
graciously provided by Pfizer Pharmaceuticals (Kalamazoo, MI).

RESULTS

Rapid effects of aldosterone on myosin light-chain phos-
phorylation. Aldosterone mediated dose- and time-dependent
MLC20 phosphorylation (Fig. 1). A significant increase in
MLC20 phosphorylation was first evident at 10 min (Fig. 1A).
Notably, after 1 h of exposure, the extent of phosphorylation had
decreased to levels not significantly greater than baseline
[analogous to the biphasic temporal effects seen for other
myosin light-chain phosphorylation agonists (22)]. Maximal
aldosterone-mediated effects were comparable to those ob-
erved after 30 min of phenylephrine treatment (127 ± 3% of
control, n = 3).

Effects of spironolactone, eplerenone, and PI3-kinase inhibi-
tion on aldosterone-mediated myosin light-chain phosphoryla-
tion. The ability of aldosterone to increase MLC20 phos-
phorylation was significantly attenuated with spironolactone

Fig. 1. Aldosterone enhances myosin light-chain (MLC) phosphorylation. A:
dose-dependent phosphorylation of 20-kDa MLC (MLC20) by aldosterone.
HITC6 cells were incubated with increasing doses of aldosterone (0.1–100
nM) for 30 min. B: temporal effects of aldosterone (100 nM) on MLC20
phosphorylation. Data represent means ± SE from 3–12 independent experi-
ments performed under identical conditions. * P < 0.05 vs. vehicle control-
treated cells by one-way ANOVA followed by Dunnett’s multiple comparison
tests.
pretreatment (Fig. 2). Pretreatment of smooth muscle cells with spironolactone alone (10 μM for 10 min) did not significantly alter the MLC20 phosphorylation ratio (Fig. 2). Similar results were obtained with the more MR-selective antagonist eplerenone, where pretreatment significantly reduced aldosterone-mediated MLC20 phosphorylation (Fig. 2), whereas pretreatment with eplerenone alone did not significantly alter the MLC20 phosphorylation ratio (Fig. 2). Also, the PI3-kinase inhibitor LY-294002 significantly attenuated aldosterone-mediated MLC20 phosphorylation (Fig. 3). In contrast, LY-303511 (the inactive analog of LY-294002) did not significantly alter aldosterone-mediated enhancement of MLC20 phosphorylation (Fig. 3). LY-294002 alone did not significantly alter the MLC20 phosphorylation ratio (Fig. 3).

Effect of aldosterone on MYPT1 phosphorylation. As noted above, the phosphorylation of the myosin phosphatase MYPT1 [either at Thr696 or Thr850 (rats)/Thr853 (humans)] results in enzyme inhibition and has been identified as a critical regulatory process in contraction (41). To determine whether aldosterone might mediate enhanced myosin light-chain phosphorylation via MYPT1 phosphorylation and/or inhibition of phosphatase function, we examined the phosphorylation status of MYPT1 after aldosterone treatment. No significant alteration in phosphorylation of MYPT1 at Thr696 was observed after 5, 10, or 30 min of aldosterone (100 nM) treatment (5 min: 97 ± 11% of control; 10 min: 96 ± 13% of control; 30 min: 103 ± 16% of control, n = 7 for all). In contrast, phenylephrine (10 μM) significantly increased Thr696 phosphorylation of MYPT1 (152 ± 16% of control, n = 7; P < 0.05), suggesting that the phosphorylation of MYPT1 in response to contractile stimuli is intact. Notably, we were unable to detect any evidence of Thr853 phosphorylation of MYPT1 under basal or stimulated conditions with either aldosterone or phenylephrine (data not shown).

Rapid effects of estrogen and hydrocortisone on myosin light-chain phosphorylation. To determine whether other steroid hormones mimicked the increase in myosin light-chain phosphorylation by aldosterone, HITC6 cells were incubated with increasing doses of either estradiol or hydrocortisone. Similar to the effect of aldosterone, both hydrocortisone and estradiol mediated significant dose-dependent increases in MLC20 phosphorylation (Fig. 4). Interestingly, both estradiol- and hydrocortisone-mediated increases in MLC20 phosphorylation were significantly attenuated after spironolactone pretreatment (Fig. 5). However, eplerenone pretreatment did not block the phosphorylation of myosin light chain by either estradiol (91 ± 8% of estradiol alone, n = 3) or hydrocortisone (115 ± 9% of hydrocortisone alone, n = 3).

Aldosterone-mediated vascular smooth muscle cell contraction. To determine whether rapid effects on myosin light-chain phosphorylation paralleled functional responses, we examined the effect of aldosterone on the contraction of HITC6 smooth muscle cells. Aldosterone mediated a dose-dependent contraction in HITC6 cells with effects seen at concentrations of 0.1 nmol/l and higher (Fig. 6). To assess whether a steroid receptor antagonist could antagonize this effect of aldosterone, we utilized spironolactone. Notably, pretreatment with higher concentrations (1, 10, or 100 μM) of spironolactone significantly induced vascular smooth muscle contraction (1 μM: 88 ± 2% of control, n = 18; 10 μM: 80 ± 4% of control, n = 9; 100 μM: 79 ± 5%, n = 17; P < 0.05 for all). These partial agonist effects of spironolactone have been previously well established in regards to hydrogen ion excretion (23, 28), as well as vascular Na-K-2Cl cotransport and enhancement of phenylephrine-mediated vascular contraction (17, 20). However, at a concentration of 0.1 μM, pretreatment with spironolactone alone did not significantly induce contraction of smooth muscle cells (93 ± 5% of control for 10 min. Cells were then incubated for an additional 30 min with or without the addition of aldosterone (ALDO, 100 nM). Data represent means ± SE from 3–6 experiments performed under identical conditions. *P < 0.05 vs. vehicle control-treated cells by one-way ANOVA followed by Dunnett’s multiple comparison tests.
control, \( n = 16 \)). Therefore, we utilized a concentration of spironolactone of 0.1 \( \mu \)M to assess its effect on aldosterone-mediated contraction. As depicted in Fig. 7, pretreatment with spironolactone (0.1 \( \mu \)M) significantly attenuated the extent of aldosterone-mediated contraction.

**Hydrocortisone- and estrogen-mediated vascular smooth muscle cell contraction.** Similar to the effects seen with aldosterone (and paralleling their effects on myosin light-chain phosphorylation), both hydrocortisone (at concentrations of 0.1 nmol/l and higher) and estradiol (at concentrations of 1.0 nmol/l and higher) mediated dose-dependent contraction of HITC6 cells (Fig. 8).

**DISCUSSION**

Our previous studies defined the rapid effects of aldosterone to attenuate vasoconstrictor responses via a PI3-kinase/NOS activation pathway (20). In the present study, we have characterized aldosterone’s vasoconstrictor response in isolated human vascular smooth muscle cells. The effect of aldosterone to mediate smooth muscle contraction parallels its effect to mediate myosin light-chain phosphorylation via a PI3-kinase-dependent pathway. Furthermore, the effects of aldosterone were inhibitable by the classical steroid receptor antagonist spironolactone and paralleled analogous effects of both hydrocortisone and estrogen.

As predicted from our initial studies in endothelium-denuded vascular rings, aldosterone mediated a rapid, dose-dependent increase in myosin light-chain phosphorylation, a widely used index of the “final common pathway” for contractile responses (24). This rapid effect of aldosterone on myosin light-chain phosphorylation paralleled its effect to mediate contraction of isolated vascular smooth muscle cells, confirming the functional consequence of myosin light-chain phosphorylation. These effects occurred over a time course consistent with the previously described rapid (so-called non-genomic) effects of aldosterone (12).

As in our previous studies in isolated cells and in vascular rings, the rapid effects of aldosterone were inhibitable by the “classical” steroid receptor antagonist spironolactone, as well as the selective MR antagonist eplerenone. Parenthetically,
those studies in which the nongenomic effects of aldosterone were not spironolactone inhibitable were generally associated with a PKC/Ca$^{2+}$/H11001 pathway of effect (21, 38). However, our previous study did not demonstrate any acute effects of aldosterone on calcium transients, suggesting that PKC activation was not a responsible mechanism (20). If so, this would have predicted our present finding that spironolactone inhibits the rapid aldosterone effect on myosin light-chain phosphorylation.

Paralleling the cellular mechanism underlying aldosterone’s endothelial-dependent effects, the effect of aldosterone to mediate myosin light-chain phosphorylation in vascular smooth muscle cells also appears to be PI3-kinase dependent. The dependence of PI3-kinase activation in mediating the rapid actions of aldosterone have been demonstrated in several models, including its effects on sodium transport (21, 35) as well on vasoconstrictor reactivity (36). Notably, functionally antagonistic effects of PI3-kinase activation in endothelial cells (mediating vasodilation) vs. vascular smooth muscle cells (mediating vasoconstriction) have been previously described (5). The effects of aldosterone would seem to follow this pattern.

Enhancement of myosin light-chain phosphorylation has been conventionally considered to be the consequence of enhanced kinase activity and/or inhibition of phosphatase activity. On the basis of our study, inhibition of phosphatase activity (at least via phosphorylation of MYPT1) does not appear to be an important mechanism of aldosterone-mediated contraction.

Both MR-dependent and MR-independent mechanisms have been suggested to be important in the rapid cardiovascular effects of aldosterone. This variability in aldosterone-mediated responses has been suggested to be due to the presence of multiple signaling pathways (14). Rapid effects of aldosterone on cAMP levels and intracellular calcium transients are maintained in MR-knockout mice (15). However, eplerenone has been reported to inhibit the rapid effects of aldosterone on p38 MAP kinase (6), Na/H exchange, calcium transients (notwithstanding the findings in MR-knockout mice above), and vasoconstriction (21). Although these findings may be viewed as contradictory, in the absence of unambiguous identification of the “MR-independent” binding site for aldosterone’s rapid effect, the selectivity of eplerenone (which was established primarily relative to its effects on other known classical steroid receptors) at this putative “alternate” site is unclear.

In our studies, both estrogen receptor and glucocorticoid receptor agonists (i.e., estradiol and hydrocortisone) mimicked the effects of aldosterone to both mediate increased MLC$_{20}$ phosphorylation and mediate smooth muscle cell contraction. As with aldosterone, both vasoconstrictor and vasodilator effects have been variably described for both estrogens and glucocorticoids (34, 40). Furthermore, a biphasic effect of estrogen on contraction has also been described, an effect that is NOS dependent (40). Also, PI3-kinase activation seems to be a common mechanism underlying the rapid effects of aldosterone as well as of estrogens and glucocorticoids (32, 33). We cannot differentiate whether this might reflect the action of multiple hormones acting on multiple, different receptors (whether classical or membrane-associated MR/glucocorticoid receptor/estrogen receptor) vs. a single receptor. On one hand, it is notable that in our study there were comparable maximal

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**Fig. 7.** Spironolactone attenuates aldosterone-mediated contraction. Vascular smooth muscle cells were pretreated with spironolactone (0.1 μM) or vehicle control for 30 min, and contraction was induced by the subsequent addition of aldosterone (100 nM), assessed over the subsequent 5 min. Spironolactone significantly attenuated aldosterone-mediated contraction. Data represent means ± SE from 3 independent experiments performed under identical conditions. *P < 0.05 vs. aldosterone alone by unpaired Student’s t-test with Welch’s correction.

**Fig. 8.** Both hydrocortisone and estradiol mediate dose-dependent contraction of vascular smooth muscle cells. HITC6 cells were incubated with increasing doses of either hydrocortisone (A) or estradiol (B) added at 5-min intervals. Data represent means ± SE from 3–4 independent experiments performed under identical conditions. *P < 0.05 vs. control-treated cells by one-way ANOVA followed by Dunnett’s multiple comparison tests.

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responses of these hormones with a broad range of potencies (i.e., aldosterone > hydrocortisone = estrogen). Furthermore, the effects of hydrocortisone and estrogen were comparably inhibited by spironolactone. These findings would be consistent with a common mechanism of effect (and potentially a single binding site), based on the well-established cross-reactivity of glucocorticoids and mineralocorticoids to commonly act on the classical MR (2) and in their commonalities in mediating the rapid effects of steroids on sodium transport (13). On the other hand, only aldosterone-mediated responses, and not estrogen- or hydrocortisone-mediated responses, were inhibited by pretreatment with the more specific MR antagonist eplerenone, suggesting that these steroids act at distinct binding sites (notwithstanding the potential ambiguity as discussed above regarding eplerenone’s effect on rapid aldosterone responses at putative non-MR sites).

Thus the issue of whether the effects of steroids on vascular contractile function are wholly mediated by multiple distinct “classical” steroid receptors or a single site must be viewed as a “work-in-progress.”

In summary, these studies demonstrate that the vasoconstrictor effects of aldosterone can be identified in human vascular smooth muscle cells. This contractile effect parallels phosphorylation of myosin light chains and is mimicked by both glucocorticoids and estrogens. Based on these findings (and previous studies), we would suggest that the functional vascular effects of aldosterone and other vasoactive steroids reflect a balance between their vasoconstrictor and vasodilator effects. Tipping this balance, as in settings of endothelial dysfunction, may prove to be an important mechanism by which aldosterone (and other steroids) may mediate its pathological effects in essential hypertension.

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