Mineralocorticoid Receptor Activation Promotes Vascular Cell Calcification

Iris Z. Jaffe, Yin Tintut, Brenna G. Newfell, Linda L. Demer, Michael E. Mendelsohn

Objective—Clinical studies demonstrate that mineralocorticoid receptor (MR) antagonism improves outcomes in cardiovascular patients and that vascular calcification correlates with adverse cardiac events. We have recently demonstrated that human vascular smooth muscle cells (VSMCs) express functional MRs that, in response to aldosterone, modulate expression of osteogenic genes including alkaline phosphatase (ALP) and bone morphogenetic protein-2 (BMP2). This study examines the effects of MR activation by aldosterone on the process of in vitro vascular calcification.

Methods and Results—Using immunoblotting and adenoviral promoter-reporter assays, we demonstrated that calcifying vascular cells (CVCs), an in vitro model of vascular calcification, express MRs that mediate both aldosterone- and cortisol-stimulated gene transcription. In this model, aldosterone stimulated ALP activity, an early marker of osteoblastic differentiation, as well as mineralization. Aldosterone antagonism with spironolactone abolished both effects implicating CVC MRs in the mechanism of aldosterone-stimulated vascular calcification. Inhibition of BMP2 signaling by overexpression of dominant negative BMP2 receptor did not attenuate aldosterone-induced osteoblastic differentiation.

Conclusions—Aldosterone activation of MR promotes osteoblastic differentiation and mineralization of VSMCs independent of BMP2 signaling. These data provide a mechanistic link between hormone-mediated VSMC MR activation and vascular calcification, two processes associated with increased risk of cardiovascular ischemic events in humans. (***Arterioscler Thromb Vasc Biol*. 2007;27:799-805.)**

Key Words: aldosterone ■ mineralocorticoid receptor ■ VSMC ■ vascular calcification ■ spironolactone

Ischemic vascular disease remains the leading cause of mortality in the developed world. In clinical trials, aldosterone antagonists, such as spironolactone and eplerenone, improve cardiovascular outcomes and prevent ischemic events in cardiovascular patients.[1,2] The beneficial effects of aldosterone antagonists on cardiovascular outcomes are much greater than predicted based on their modest blood pressure lowering effects, suggesting a direct role for aldosterone in regulating vascular function. However, the mechanism for the beneficial role of aldosterone antagonists in clinical trials is not well characterized at a molecular level.

Aldosterone acts by binding to the mineralocorticoid receptor (MR), an intracellular steroid hormone receptor that functions as a ligand-activated transcription factor.[3] The mineralocorticoid aldosterone and the glucocorticoid cortisol both bind to human MR with equal affinity.[3] Plasma glucocorticoid concentrations are 100- to 1000-fold higher than those of aldosterone. However, MRs in aldosterone-responsive tissues are able to respond to aldosterone because these tissues express the enzyme 11-β-hydroxysteroid dehydrogenase type 2 (11βHSD2), that converts cortisol to its 11-keto derivatives, which have a low affinity for MR.[4] Aldosterone antagonists act by competitively inhibiting aldosterone binding to MR.[3] Antagonists like spironolactone inhibit binding of both aldosterone and cortisol to MR but are receptor specific because they do not inhibit steroid hormone binding to the glucocorticoid receptor (GR) (Arriza et al[3] and Jaffe IZ, unpublished data, 2004). In the kidney, an aldosterone responsive tissue, aldosterone-activated MR stimulates expression of genes involved in sodium and water absorption, thereby maintaining and, in some pathological conditions, elevating blood pressure.[5] Animal studies also suggest a direct effect of aldosterone on vascular function that is independent of renal-mediated blood pressure effects.[6–8]

Recent studies have begun to explore the molecular mechanisms of the direct actions of aldosterone on the cardiovascular system. We and others have demonstrated the expression of functional MRs and 11βHSD2 in the heart, large arteries, VSMCs, and endothelial cells.[9–13] In addition, we have recently demonstrated that in human coronary artery

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SMCs, aldosterone regulates the expression of several genes that have been implicated in vascular calcification including the bone morphogenetic protein-2 (BMP2) and the alkaline phosphatase (ALP) genes.[13]

The presence of vascular calcification in humans, as demonstrated by x-ray and, more recently, by electron beam computed tomography (EBCT), predicts the risk of new or recurrent vascular ischemic events and vascular mortality.[14–17] Thus, both aldosterone excess and vascular calcification are associated with an increased risk of cardiovascular ischemia in humans and our recent expression data in human coronary artery SMCs suggests a possible link between these processes.[13]

Vascular calcification, which occurs at sites of atherosclerosis, is a regulated process that resembles the process of osteoblastic differentiation and biomineralization that occurs during bone development (reviewed in[18–20]). This process involves transcription of genes involved in osteoblastic differentiation including the ALP gene. Subpopulations of human and bovine aortic SMCs have been found to spontaneously calcify in vitro.[21] These calcifying vascular cells (CVCs) have been extensively studied as a model of in vitro vascular calcification and have been shown to recapitulate many of the pathways that regulate osteoblastic differentiation in bone and, during this process, to upregulate many of the proteins present in calcified vascular lesions in human atherosclerotic vessels.[21–24] In vitro calcification of CVCs is regulated by many of the factors involved in bone development and atherosclerosis progression including: steroid hormones, oxidized lipids, bone morphogenetic proteins (specifically BMP2), and inflammatory cytokines (reviewed in[19,25]).

Thus, in this study, we use calcifying vascular cells (CVCs) as a model to test the hypothesis that aldosterone promotes in vitro vascular calcification and that this occurs via activation of mineralocorticoid receptors in these cells. We demonstrate that aldosterone stimulates osteogenic differentiation and mineralization of vascular cells. This process is mediated by functional MRs in CVCs independent of BMP2 signaling.

Methods

Reagents, Cell Lines, and Culture Techniques

Reagents: aldosterone, spironolactone, cortisol (Research Plus, Inc, dimethylsulfoxide [DMSO] vehicle), and glycerrhetinic acid (Masco Worldwide, ethanol vehicle). Equal amounts of vehicle were used in all experiments. Cell lines: immortalized human coronary artery and aortic SMCs[13] (passage 11 to 16) and calcifying vascular cells (CVCs) from bovine aortic SMCs[26](passage 15 to 24). Before each experiment, the media were changed from 15% fetal bovine serum (FBS) to 10% FBS (Figures 3 [left panel] and 5) or 15% charcoal stripped FBS (Figures 1, 2, 3 [right panel], and 4) as indicated, to reduce the effect of endogenous ligands.

Immunoblotting

Cell lysates were prepared and immunoblotted as described.[13] Three uL of lysate pellet or supernatant from HEK293 cells overexpressing MR or 11βHSD2 was loaded in the first lane(s) as a positive control followed by lysate from VSMCs and/or bovine kidney as indicated. Antibodies: polyclonal MR (Santa Cruz, 1:200), 11βHSD2 (Alpha Diagnostic International, 1:2000), and monoclonal beta actin (Sigma, 1:10 000).

Adenovirus Infection and Luciferase Reporter Assay

CVCs and aortic VSMCs were transduced with an adenoviral MRE-luciferase reporter containing the mouse mammary tumor virus (MMTV) promoter that includes multiple MR response elements (MREs).[13] Indicated concentrations of vehicle, ligand, and/or inhibitor were added for 18 hours. Cells were lysed and luciferase activity was determined in duplicate as described.[13] Each experiment was repeated at least 3 times and is expressed as mean fold activation compared with vehicle control +SEM.

Alkaline Phosphatase Assay

CVCs were seeded in 48-well plates at 20 000 cells per well in DMEM supplemented with 15% FBS. One day after seeding, cells were treated with agents in DMEM supplemented with either 10% normal serum or 15% charcoal-stripped serum, as indicated. In Figure 4, the cells were infected for 3 hours in 0.1% charcoal-stripped serum with BMP2 receptor dominant negative (BMP2RDN) virus (HA-Alk6, a generous gift from K. Miyazono and M. Fujii, University of Tokyo, Japan[27]) or control GFP containing virus. Fresh media with agents were then added every 3 to 4 days. ALP activity from whole cell lysates was assayed in quadruplicate and normalized to total protein (Bradford assay) as described.[26]

Mineralization Assays

CVCs were seeded as described above and treated with agents 1 day after plating in DMEM supplemented with 10% normal serum. At the first and successive media changes, β-glycerophosphate (5 mmol/L) was added along with fresh agents. After 8 to 11 days, mineral incorporation was quantified in quadruplicate by the *o*-cresolphthalein complexone method as described[28] and normalized to total protein (Bradford assay). Von Kossa staining for calcium mineral was performed as described.[29]

Statistical Analysis

Each experiment was repeated at least 3 times, and data are expressed as mean±SD (unless otherwise indicated). Means were compared using one-way ANOVA, with between group comparisons by Student-Newman-Keuls method (Figures 1, 2, and 4) or Fisher protected least significant difference test (Figures 3 and 5). A value of $P<0.05$ was considered significant.

Results

CVCs Express Functional Mineralocorticoid Receptors

CVCs are a subset of primary bovine aortic smooth muscle cells that form nodules and incorporate calcium mineral in their extracellular matrix.[29] To use this in vitro model to investigate the role of aldosterone in the process of VSMC calcification, we began by exploring whether CVCs express mineralocorticoid receptors (MR). Immunoblotting with MR-specific antibody demonstrated the presence of MR protein in cell lysates from CVCs (Figure 1A), predominantly in the soluble fractions, as previously described in human coronary SMCs.[13] CVCs expressed amounts of MR similar to that in human coronary and aortic SMCs in culture (Figure 1A, lanes 3 versus 5 and 7). Next, we tested the functional activity of CVC MRs. CVCs were infected with a MRE-containing adenovirus promoter-reporter construct and treated with hormones. CVC MRs mediated ligand-dependent transcriptional activation in response to aldosterone that was completely inhibited by the MR antagonist spironolactone (Figure 1B, left). When the dose response relationship was compared with that of noncalcifying human aortic SMCs (Figure 1B, right), CVC MRs required higher doses of aldosterone to activate

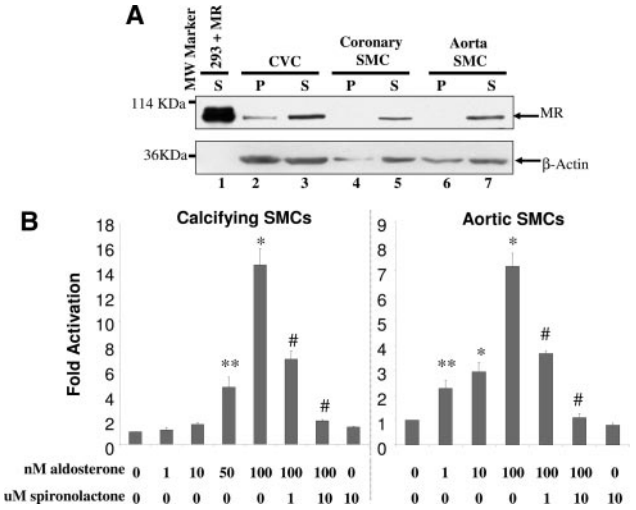

Figure 1. MR expression and transcriptional activation by aldosterone in CVCs. A, Immunoblot with anti-MR or anti–β-actin antibodies demonstrating MR protein in control 293 cells overexpressing MR, CVCs, human coronary, and human aortic SMCs. P indicates pellet; S, supernatant. One of three similar studies is shown. B, Aldosterone dose response of MR-mediated gene transcription in CVCs compared with aortic SMCs. Cells were transduced with the MRE-Luciferase reporter adenovirus. Luciferase activity at each hormone dose is expressed as mean fold activation over vehicle alone. *$P<0.001$ vs no aldosterone, ** $P<0.05$ vs no aldosterone, # $P<0.001$ vs 100 nmol/L aldosterone.

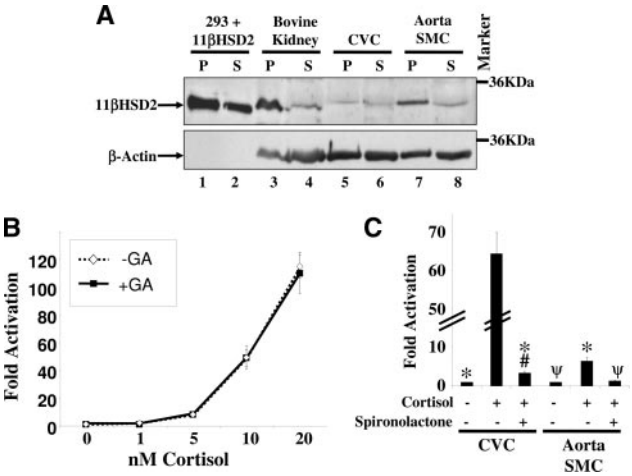

Figure 2. 11βHSD2 expression and MR activation by corticosteroids in CVCs. A, Immunoblot with anti-11βHSD2 or anti–β-actin antibodies demonstrating 11βHSD2 protein in 293 cells overexpressing 11βHSD2, bovine kidney, CVCs, and aortic SMCs. P indicates pellet; S, supernatant. One of three similar studies is shown. B, Lack of 11βHSD2 effect on MRE activation in CVCs. Activation of the MRE-Luciferase reporter adenovirus was measured at each dose of cortisol in the presence of vehicle or 10 μmol/L glycerrhetinic acid. C, Cortisol activation of MRE is MR specific. In CVCs and aortic SMCs, cortisol activation of the MRE-Luciferase adenovirus is measured in the presence and absence of spironolactone. *$P<0.05$ vs CVC+Cortisol, #$P<0.05$ vs CVC control, ψ$P<0.05$ vs Aorta+Cortisol.

transcription (CVC EC_{50}=71 nmol/L, Aorta SMC EC_{50}=36 nmol/L). Figure 1B is also the first demonstration of aldosterone-stimulated MR-mediated gene transcription in human aortic SMCs.

11βHSD2 Expression and Corticosteroid Responsiveness of MR in CVCs

Next, the presence of the cortisol-inactivating enzyme 11βHSD2 was explored in CVCs. In Figure 2A, immunoblot with 11βHSD2 antibody demonstrated that CVCs express a small amount of 11βHSD2 enzyme (lanes 5 and 6), less than in aortic SMCs (lanes 7 and 8). Positive controls demonstrate that the polyclonal antibody recognizes both human 11βHSD2 (overexpressed in 293 cells, lanes 1 and 2) and bovine 11βHSD2 (bovine kidney, lanes 3 and 4). Consistent with the low expression level in CVCs, cortisol-induced luciferase activity from the MRE-containing reporter virus was not further enhanced by glycerrhetinic acid (GA), an inhibitor of 11βHSD2 (Figure 2B). The same concentration of GA (10 μmol/L) simultaneously inhibited 11βHSD2 activity in other vascular cells (data not shown). This suggested that MRs in CVCs may be more sensitive to activation by cortisol. This hypothesis was confirmed using 10 nmol/L cortisol in the adenoviral promoter-reporter assay resulting in 10-fold greater reporter activity in CVCs (65-fold) compared with aortic SMCs (6.4-fold; Figure 2C). This effect was virtually abolished by cotreatment with spironolactone suggesting that it is mediated almost entirely by MR in these cells, not GR. From Figure 1 and 2 we conclude that CVCs contain MRs that function as transcriptional regulators that can be activated by both aldosterone and cortisol. In the remaining experiments, we chose to use aldosterone, a MR specific agonist.

Aldosterone Stimulates Alkaline Phosphatase Activity in a MR-Dependent Manner

The effect of aldosterone on alkaline phosphatase (ALP) activity, an early marker of osteoblastic differentiation, was next tested. After 7 days of aldosterone treatment of CVCs in normal serum, ALP activity in CVCs was slightly but significantly enhanced, and this effect was blocked by the MR antagonist spironolactone (Figure 3, left). Spironolactone treatment also attenuated basal ALP activity, supporting that basal mineralocorticoid activity exists in CVCs cultured in serum. To remove potential effects of endogenous aldosterone and other MR ligands on ALP activity, these experiments were repeated using media supplemented with charcoal-stripped serum. In the stripped serum, aldosterone treatment enhanced ALP activity 3-fold, and simultaneous treatment with spironolactone prevented the aldosterone-induced increase in ALP activity (Figure 3, right). These data support that normal, unstripped serum contains MR agonists that activate ALP activity in CVCs, and that enhancement of ALP activity by aldosterone in this assay is mediated by the MR. Time course experiments also demonstrated that ALP activation by aldosterone was significantly increased at 7 days and the activation persisted through 17 days of aldosterone treatment (data not shown). At each time point tested, the effect was inhibited by spironolactone (data not shown) confirming that this is a MR-mediated effect.

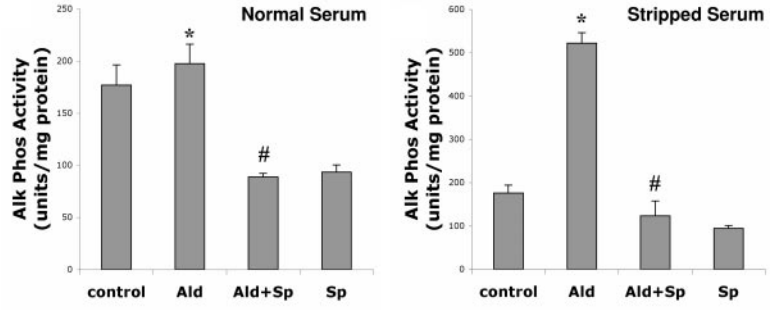

Figure 3. Aldosterone stimulates alkaline phosphatase activity in CVCs in a MR-dependent manner. Alkaline phosphatase activity of whole cell extracts from cells treated for 7 days with vehicle, aldosterone (Ald,10^{-7} mol/L) and/or spironolactone (Sp,10^{-5} mol/L), as indicated, in normal serum (*$P<0.05$ vs control, #$P<0.0001$ vs Ald) or in charcoal-stripped serum (*$P<0.0001$ vs control, #$P<0.0001$ vs Ald).

Aldosterone Stimulation of Alkaline Phosphatase Activity Is Independent of BMP2 Signaling

Our previous gene profile data from human coronary artery SMCs revealed that expression of both the ALP and the BMP2 genes was upregulated by aldosterone.[13] Quantitative RT-PCR time course experiments revealed that BMP2 expression was upregulated rapidly (3-fold after 1 hour of aldosterone treatment, back to basal expression levels by 4 hours) whereas aldosterone-stimulated ALP gene expression began later (no change at 1 or 4 hours and a 2-fold increase after 24 hours) (Jaffe and Mendelsohn[13] and data not shown), suggesting a possible cascade effect. Thus, we next tested the hypothesis that the BMP2 signaling may mediate aldosterone activation of ALP activity in CVCs. BMP2 activates ALP gene expression by binding to the Alk6 type-I BMP receptor resulting in phosphorylation of SMAD transcription factors that stimulate osteogenic gene expression.[30] We infected CVCs with a virus containing a dominant negative (DN, kinase-inactivated) Alk6 BMP2 receptor (BMP2RDN). Immunoblotting of infected CVC lysates with an antibody to the tagged DN receptor demonstrated a high level of expression at 24 hours that persisted for 7 days (data not shown). After 7 days of treatment with aldosterone, stimulation of ALP activity in these cells was compared with cells infected with GFP containing control virus. The BMP2RDN virus significantly inhibited basal ALP activity consistent with inhibition of Alk6 BMP receptors in infected cells (Figure 4, compare bars 1 and 3). Addition of aldosterone resulted in a similar increase in ALP activity in BMP2RDN-expressing cells compared with control virus cells suggesting that this pathway is not necessary for aldosterone-stimulated osteoblastic differentiation. From Figure 3 and 4, we concluded that aldosterone stimulates ALP activity in CVCs by activating endogenous MRs via a mechanism that is independent of the BMP2 osteogenic pathway.

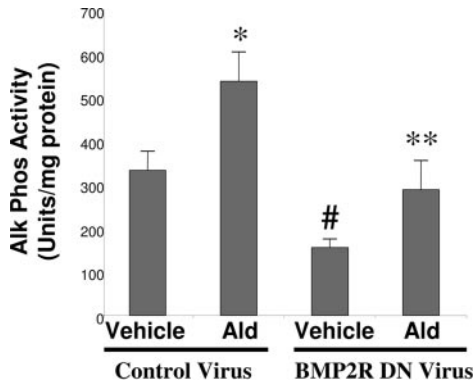

Figure 4. Aldosterone stimulates alkaline phosphatase activity independent of BMP2 signaling. ALP activity of whole cell extracts from CVCs expressing BMP2R DN for 7 days in charcoal-stripped serum. *$P<0.005$ vs control virus vehicle, #$P<0.05$ vs control virus vehicle, **$P<0.05$ vs BMP2R DN virus vehicle.

Aldosterone Stimulates Vascular Cell Mineralization by MR Activation

To explore whether aldosterone influences VSMC mineralization, CVCs were treated with aldosterone for 10 days in the presence of β-glycerophosphate and mineralization was assessed by the *o*-cresolphthalein complexone method.[28] Aldosterone enhanced CVC mineralization in a dose-dependent manner (Figure 5A) beginning at 10 nmol/L. This threshold concentration of aldosterone is consistent with those reported previously to activate MR-mediated gene transcription in VSMCs in culture[13] and with aldosterone levels measured in patients with congestive heart failure or coronary artery disease.[31,32] Aldosterone-stimulated CVC mineralization was inhibited by cotreatment with spironolactone as measured by 2 independent methods (Figure 5B and 5C) supporting that mineralization attributable to aldosterone is mediated by MR activation. In addition, basal levels of mineralization were significantly inhibited by spironolactone alone (Figure 5B and 5C), supporting that basal activation of mineralization in the presence of serum derives at least in part from MR ligands in normal serum. As they calcify, CVCs aggregate into nodules that consist of cells and extracellular matrix.[33] In Figure 5C, aldosterone appears to increase nodule formation (arrow). When quantified, aldosterone significantly increased CVC nodule formation by 20% to 30% above controls after 18 days of treatment, and this effect was reversed by simultaneous treatment with spironolactone (data not shown). In addition, when CVCs were pretreated with noggin, an exogenous BMP2 inhibitor, at concentrations previously demonstrated to inhibit BMP2 signaling in CVCs,[34] aldosterone still significantly stimulated CVC mineralization (data not shown). Thus, aldosterone stimulates osteogenic differentiation and mineralization of vascular cells in a MR-dependent manner through a pathway that is independent of BMP2 signaling.

Discussion

Vascular calcification and mineralocorticoid receptor activation are two processes that have been independently associ-

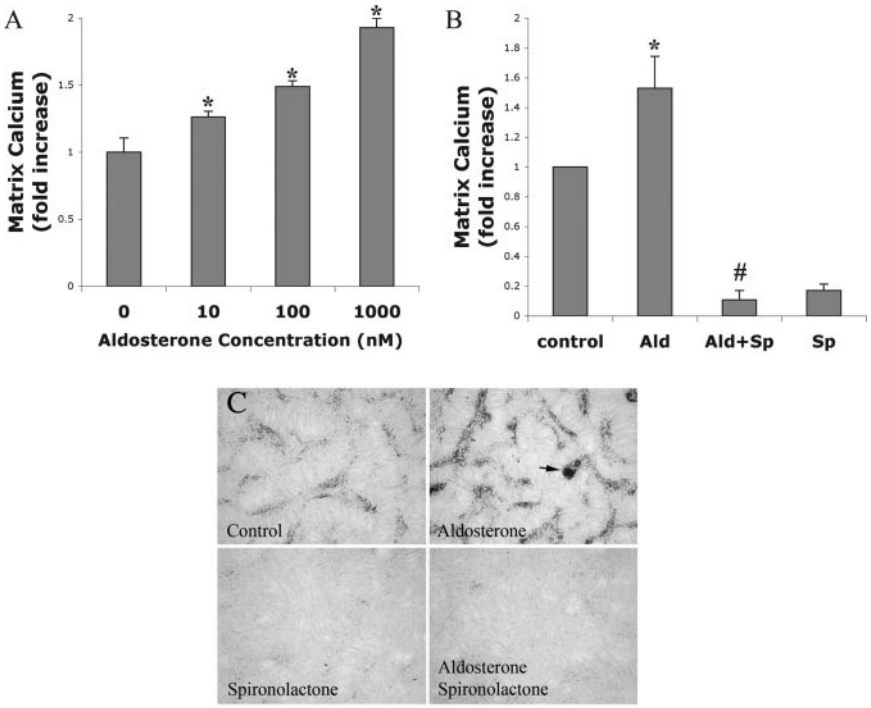

Figure 5. Aldosterone activation of MR stimulates vascular cell calcification. A, Matrix calcium incorporation in response to indicated concentrations of aldosterone. *$P<0.0001$ vs control. B, Matrix calcium incorporation (*$P<0.0005$ vs control, #$P<0.0001$ vs Ald) and (C) Von Kossa staining (which identifies calcium mineral as black, arrow) of CVCs treated for 10 days with vehicle control, aldosterone (Ald, 10^{-7} mol/L) or spironolactone (Sp, 10^{-5} mol/L), as indicated. Magnification: ×40.

ated with atherosclerotic vascular disease in humans and predict the occurrence of ischemic events and cardiovascular mortality.[1,2,14–17] We have recently demonstrated that human coronary SMCs express functional MRs and, in response to aldosterone, express genes known to be involved in vascular calcification[13] suggesting a potential mechanistic link between these two clinically relevant processes. Here we use calcifying vascular cells, a subset of pericyte-like primary bovine aortic SMCs that spontaneously develop an osteogenic phenotype,[22] to explore the role of MR activation in the process of in vitro vascular calcification. We report for the first time that aldosterone stimulates osteogenic differentiation and calcification of CVCs, effects abolished in the presence of the MR antagonist spironolactone.

Using immunoblotting and adenoviral promoter-reporter assays, we demonstrate that CVCs express MRs capable of ligand-dependent transcriptional activation. Compared with our previous data in human coronary SMCs[13] and new data in human aortic SMCs, CVCs required higher aldosterone levels for transactivation. Recent data suggests that vascular cells may synthesize aldosterone[11] and hence local concentrations of aldosterone in vivo may be much higher than those measured in serum and may be able to activate CVCs. Alternatively, we demonstrate that CVCs express only small amounts of 11βHSD2 consistent with the demonstrated exquisite sensitivity of CVC MRs to activation by cortisol that is unaltered by 11βHSD2 inhibition. Thus, at physiological circulating concentrations of hormones, the in vivo ligand for MR in the subset of osteogenic vascular cells is likely cortisol rather than aldosterone. Others have shown that in SMCs cultured from human atherosclerotic lesions, MR expression is maintained while 11βHSD2 and GR levels are decreased.[35] These data suggest that in diseased vessels, MR may be activated by cortisol. Although cortisol can also bind GR, spironolactone inhibition of transactivation confirms that in CVCs, cortisol activates transcription by binding to MR. Our findings with cortisol are in agreement with previous reports demonstrating that the corticosteroid dexamethasone stimulates CVC differentiation;[23,36] however, the potential role of the MR in mediating this process has not been previously appreciated and remains to be tested in future experiments.

Alkaline phosphatase activity is an early marker of osteoblastic differentiation. We demonstrate that in CVCs, aldosterone increases ALP activity in a MR-dependent manner. The effect of aldosterone on ALP activity is more pronounced when charcoal stripped media is used to remove endogenous steroid ligands and is consistent with the inhibition of basal ALP activity that we see with spironolactone in complete serum. These data support an activating effect of endogenous MR ligands in normal, unstripped serum. Thus, aldosterone, via MR activation, promotes osteoblastic differentiation of VSMCs in vitro and the level of MR ligands present in normal serum is sufficient to promote this process.

Our gene profiling data in human coronary artery SMCs demonstrated aldosterone upregulation of ALP gene expression at 24 hours consistent with direct MR binding to the ALP promoter or indirect activation through rapid induction of BMP2.[13] BMP2 expression is elevated at sites of calcific arteriopathy,[37] and it functions as a regulator of early stages of vascular calcification.[29] Hence we considered the possibility that aldosterone-stimulated BMP expression regulates ALP activity in CVCs. However, we found that inhibition of BMP2 signaling with a dominant negative BMP2 receptor did not abolish aldosterone-stimulated ALP activity suggesting that MR stimulates ALP activity by a BMP2-independent mechanism. Future study is warranted to investigate the mechanism by which aldosterone and MR regulate the ALP promoter in vascular cells.

We have also demonstrated that aldosterone promotes nodule formation and mineralization of CVCs in culture. Animal models suggest that aldosterone may play a role in VSMC proliferation and migration,[6,38] and the role of these processes in nodule formation and subsequent mineralization remains to be investigated. Treatment of CVCs with noggin to inhibit BMP2 binding to its receptor did not inhibit aldosterone-stimulated mineralization. However, spironolactone treatment prevented aldosterone-stimulated mineralization, thus it is clear that this process is mediated by MR activation in these cells. Whether vascular calcification is simply a marker of vascular disease or plays a direct role in atherosclerotic plaque stability and risk of plaque rupture is not known. Clinical studies using intravascular ultrasound (IVUS) demonstrate that the extent and pattern of vascular calcification differs in patients with acute plaque rupture versus chronic stable angina,[39,40] suggesting a direct role for vascular calcification in acute coronary events. Because aldosterone, in animal models and human pathological conditions, stimulates atherosclerosis and cardiovascular ischemic events, it will be important to determine whether aldosterone-induced changes in SMC architecture (nodule formation) and mineralization in vivo result in altered atherosclerotic plaque stability. This may begin to elucidate the molecular mechanisms by which aldosterone antagonists prevent cardiac ischemic events in human clinical trials.

In summary, aldosterone promotes osteoblastic differentiation and mineralization of calcifying vascular cells in a MR-dependent manner that is independent of BMP2 signaling. MR in calcifying VSMCs is particularly sensitive to activation by glucocorticoids. These observations suggest that one of the clinical effects of MR antagonists in humans may be inhibition of glucocorticoid-activated, MR-mediated, vascular calcification. This is particularly important in patients treated with chronic high dose corticosteroids for inflammatory diseases such as lupus and rheumatoid arthritis (RA). These patients demonstrate accelerated atherosclerosis and increased coronary artery calcification.[41,42] The extent of atherosclerosis and medial arterial calcification in these patients correlates with glucocorticoid exposure independent of traditional cardiac risk factors, measures of inflammation, and inflammatory disease severity.[41–43] Because cardiovascular disease remains the leading cause of death in this patient population, it would be important to test the potential role of MR antagonists in preventing adverse cardiovascular outcomes in these patients.

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Disclosures

None.

References

1. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J, Wittes J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N Engl J Med.* 1999;341:709–717.
2. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, Bittman R, Hurley S, Kleiman J, Gatlin M. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *N Engl J Med.* 2003;348:1309–1321.
3. Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science.* 1987;237:268–275.
4. Farman N, Bocchi B. Mineralocorticoid selectivity: Molecular and cellular aspects. *Kidney International.* 2000;57:1364–1369.
5. Rogerson FM, Fuller PJ. Mineralocorticoid action. *Steroids.* 2000;65:61–73.
6. Van Belle E, Bauters C, Wernert N, Hamon M, McFadden EP, Racadot A, Dupuis B, Lablanche JM, Bertrand ME. Neointimal thickening after balloon denudation is enhanced by aldosterone and inhibited by spironolactone, and aldosterone antagonist. *Cardiovasc Res.* 1995;29:27–32.
7. Rajagopalan S, Duquaine D, King S, Pitt B, Patel P. Mineralocorticoid receptor antagonism in experimental atherosclerosis. *Circulation.* 2002;105:2212–2216.
8. Rocha R, Funder JW. The pathophysiology of aldosterone in the cardiovascular system. *Ann N Y Acad Sci.* 2002;970:89–100.
9. Funder JW, Pearce PT, Smith R, Campbell J. Vascular type I aldosterone binding sites are physiological mineralocorticoid receptors. *Endocrinol.* 1989;125:2224–2226.
10. Lombes M, Oblin ME, Gasc JM, Baulieu EE, Farman N, Bonvalet JP. Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. *Circ Res.* 1992;71:503–510.
11. Slight SH, Joseph J, Ganjam VK, Weber KT. Extra-adrenal mineralocorticoids and cardiovascular tissue. *J Mol Cell Cardiol.* 1999;31:1175–1184.
12. Kayes-Wandover KM, White PC. Steroidogenic enzyme gene expression in the human heart. *J Clin Endocrinol Metab.* 2000;85:2519–2525.
13. Jaffe IZ, Mendelsohn ME. Angiotensin II and aldosterone regulate gene transcription via functional mineralocortocoid receptors in human coronary artery smooth muscle cells. *Circ Res.* 2005;96:643–650.
14. Kondos GT, Hoff JA, Sevrukov A, Daviglus ML, Garside DB, Devries SS, Chomka EV, Liu K. Electron-beam tomography coronary artery calcium and cardiac events: A 37-month follow-up of 5635 initially asymptomatic low- to intermediate-risk adults. *Circulation.* 2003;107:2571–2576.
15. Raggi P, Callister TQ, Cooil B, He ZX, Lippolis NJ, Russo DJ, Zelinger A, Mahmarian JJ. Identification of patients at increased risk of first unheralded acute myocardial infarction by electron-beam computed tomography. *Circulation.* 2000;101:850–855.
16. Wilson PW, Kauppila LI, O'Donnell CJ, Kiel DP, Hannan M, Polak JM, Cupples LA. Abdominal aortic calcific deposits are an important predictor of vascular morbidity and mortality. *Circulation.* 2001;103:1529–1534.
17. Wong ND, Hsu JC, Detrano RC, Diamond G, Eisenberg H, Gardin JM. Coronary artery calcium evaluation by electron beam computed tomography and its relation to new cardiovascular events. *Am J Cardiol.* 2000;86:495–498.
18. Collin-Osdoby P. Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. *Circ Res.* 2004;95:1046–1057.
19. Abedin M, Tintut Y, Demer LL. Vascular calcification: mechanisms and clinical ramifications. *Arterioscler Thromb Vasc Biol.* 2004;24:1161–1170.
20. Vattikuti R, Towler DA. Osteogenic regulation of vascular calcification: an early perspective. *Am J Physiol Endocrinol Metabol.* 2004;286:E686–E696.
21. Parhami F, Bostrom K, Watson K, Demer LL. Role of molecular regulation in vascular calcification. *J Atheroscler Thromb.* 1996;3:90–94.
22. Bostrom K, Watson KE, Stanford WP, Demer LL. Atherosclerotic calcification: relation to developmental osteogenesis. *Am J Cardiol.* 1995;75:88B–91B.
23. Mori K, Shioi A, Jono S, Nishizawa Y, Morii H. Dexamethasone enhances In vitro vascular calcification by promoting osteoblastic differentiation of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1999;9:2112–2118.
24. Tintut Y, Patel J, Parhami F, Demer LL. Tumor necrosis factor-alpha promotes in vitro calcification of vascular cells via the cAMP pathway. *Circulation.* 2000;102:2636–2642.

25. Tintut Y, Demer LL. Recent advances in multifactorial regulation of vascular calcification. *Curr Opin Lipidol.* 2001;12:555–560.
26. Tintut Y, Parhami F, Bostrom K, Jackson SM, Demer LL. cAMP stimulates osteoblast-like differentiation of calcifying vascular cells. Potential signaling pathway for vascular calcification. *J Biol Chem.* 1998;273:7547–7553.
27. Brederlau A, Faigle R, Elmi M, Zarebski A, Sjoberg S, Fujii M, Miyazono K, Funa K. The bone morphogenetic protein type Ib receptor is a major mediator of glial differentiation and cell survival in adult hippocampal progenitor cell culture. *Mol Biol Cell.* 2004;15:3863–3875.
28. Jono S, Nishizawa Y, Shioi A, Morii H. Parathyroid hormone-related peptide as a local regulator of vascular calcification. Its inhibitory action on in vitro calcification by bovine vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1997;17:1135–1142.
29. Watson KE, Parhami F, Shin V, Demer LL. Fibronectin and collagen I matrixes promote calcification of vascular cells in vitro, whereas collagen IV matrix is inhibitory. *Arterioscler Thromb Vasc Biol.* 1998;18:1964–1971.
30. Miyazono K, Maeda S, Imamura T. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev.* 2005;16:251–263.
31. van de Wal RM, Plokker HW, Lok DJ, Boomsma F, van der Horst FA, van Veldhuisen DJ, van Gilst WH, Voors AA. Determinants of increased angiotensin II levels in severe chronic heart failure patients despite ACE inhibition. *Int J Cardiol.* 2006;106:367–372.
32. Amano T, Matsubara T, Izawa H, Torigoe M, Yoshida T, Hamaguchi Y, Ishii H, Miura M, Hayashi Y, Ogawa Y, and Murohara T. Impact of plasma aldosterone levels for prediction of in-stent restenosis. *Am J Cardiol*. 2006;97:785–788.
33. Garfinkel A, Tintut Y, Petrasek D, Bostrom K, Demer LL. Pattern formation by vascular mesenchymal cells. *Proc Natl Acad Sci U S A.* 2004;101:9247–9250.
34. Shin V, Zebboudj AF, Bostrom K. Endothelial cells modulate osteogenesis in calcifying vascular cells. *J Vasc Res.* 2004;41:193–201.
35. Bray PJ, Du B, Mejia VM, Hao SC, Deutsch E, Fu C, Wilson RC, Hanauske-Abel H, McCaffrey TA. Glucocorticoid resistance caused by reduced expression of the glucocorticoid receptor in cells from human vascular lesions. *Arterioscler Thromb Vasc Biol.* 1999;19:1180–1189.
36. Kirton JP, Wilkinson FL, Canfield AE, Alexander MY. Dexamethasone downregulates calcification-inhibitor molecules and accelerates osteogenic differentiation of vascular pericytes: implications for vascular calcification. *Circ Res.* 2006;98:1264–1272.
37. Bostrom K, Watson KE, Horn S, Wortham C, Herman IM, Demer LL. Bone morphogenetic protein expression in human atherosclerotic lesions. *J Clin Invest.* 1993;91:1800–1809.
38. Lacolley P, Labat C, Pujol A, Delcayre C, Benetos A, Safar M. Increased carotid wall elastic modulus and fibronectin in aldosterone-salt-treated rats: effects of eplerenone. *Circulation.* 2002;106:2848–2853.
39. Beckman JA, Ganz J, Creager MA, Ganz P, Kinlay S. Relationship of clinical presentation and calcification of culprit coronary artery stenoses. *Arterioscler Thromb Vasc Biol.* 2001;21:1618–1622.
40. Ehara S, Kobayashi Y, Yoshiyama M, Shimada K, Shimada Y, Fukuda D, Nakamura Y, Yamashita H, Yamagishi H, Takeuchi K, Naruko T, Haze K, Becker AE, Yoshikawa J, Ueda M. Spotty calcification typifies the culprit plaque in patients with acute myocardial infarction: an intravascular ultrasound study. *Circulation.* 2004;110:3424–3429.
41. Mok CC. Accelerated atherosclerosis, arterial thromboembolism, and preventive strategies in systemic lupus erythematosus. *Scand J Rheumatol.* 2006;35:85–95.
42. del Rincon I, O'Leary DH, Haas RW, Escalante A. Effect of glucocorticoids on the arteries in rheumatoid arthritis. *Arthritis Rheum.* 2004;50:3813–3822.
43. Raynauld JP. Cardiovascular mortality in rheumatoid arthritis: How harmful are corticosteroids? *J Rheumatol.* 1997;24:415–416.

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