Elevated Mineralocorticoid Receptor Activity in Aged Rat Vascular Smooth Muscle Cells Promotes a Proinflammatory Phenotype via Extracellular Signal-Regulated Kinase 1/2 Mitogen-Activated Protein Kinase and Epidermal Growth Factor Receptor–Dependent Pathways

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Abstract—Arterial aging is a predominant risk factor for the onset of cardiovascular diseases, such as hypertension, myocardial infarction, or stroke. Aging is associated with intravascular renin-angiotensin system activation, increased vascular stiffness, intima-media thickening, and a proinflammatory phenotype. Little is known about the influence of aldosterone on arterial aging. Hence, we hypothesized that aldosterone and mineralocorticoid receptor (MR) activation might contribute to and possibly accelerate the arterial aging process. We demonstrate increased MR expression in whole aortae and early passage aortic vascular smooth muscle cells from aged (30 months) compared with adult (8 months) F344XBN rats. Sensitivity to aldosterone-induced extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase activity is increased in aged cells. MR blockade and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase inhibition prevent age-associated increases of transforming growth factor-β, intercellular adhesion molecule 1, and procollagen 1. Aldosterone increases expression of proinflammatory marker proteins, shifting the phenotype of adult vascular smooth muscle cells toward the proinflammatory phenotype of aged rats. Epidermal growth factor receptor expression is increased with age and by aldosterone, and inhibition of epidermal growth factor receptor tyrosine kinase decreases age-associated proinflammatory marker expression. Our data support the hypothesis that increased constitutive MR signaling may promote and amplify age-associated inflammation that accompanies arterial aging through increased angiotensin II–stimulated expression of MR and enhanced sensitivity to aldosterone-mediated extracellular signal-regulated kinase 1/2 activation, likely related to increased epidermal growth factor receptor expression. (Hypertension. 2010;55:1476-1483.)

Key Words: aldosterone ▪ arterial aging ▪ vascular smooth muscle cells ▪ mineralocorticoid receptor ▪ inflammation

The number of Americans ≥65 years of age will more than double, from 34.8 million in 2000 to 70.3 million by 2030 according to US Census Bureau data. As a consequence, the United States and other developed societies will be confronted with a substantial rise in aging-associated diseases, such as cardiovascular diseases, the leading cause of death in these societies. Although there are well-known risk factors for the onset of cardiovascular disease, such as hypertension, diabetes mellitus, dyslipidemias, smoking, or a sedentary lifestyle, advancing age confers the major risk.1,2

Arterial aging is associated with increased expression and activation of the intravascular renin-angiotensin system, a proinflammatory phenotype, intimal and medial thickening, vascular stiffening, and endothelial dysfunction.3,4 The age-associated arterial inflammatory phenotype includes increased expression of chemokines, such as monocyte chemoattractant protein 1, proinflammatory transcription factors, adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), profibrotic molecules like transforming growth factor (TGF)-β, and increased type 2 matrix metalloproteinase activity.5-7 It has been hypothesized that such age-associated changes render the arteries more susceptible to pathophysiological disease mechanisms, providing the basis for a lower threshold, increased severity, and, hence, poorer prognosis of the disease.8,9 However, the exact mechanisms and potential stimuli driving the age-associated phenotype are

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still not completely understood, and no interventional strategy is known to prevent or efficiently retard arterial aging in humans.\textsuperscript{10}

While the classic role of aldosterone is to regulate water and electrolyte balance and, hence, blood pressure homeostasis, aldosterone induces structural and functional alterations in the heart, kidneys, and vessels, for example, as myocardial fibrosis, nephrosclerosis, vascular inflammation, and remodeling.\textsuperscript{11–13} Accordingly, elevated aldosterone levels are considered an independent cardiovascular risk factor. The metabolic syndrome, a cluster of cardiovascular risk factors with increased cardiovascular morbidity and mortality that increases with age,\textsuperscript{14,15} has been associated with elevated plasma aldosterone levels.\textsuperscript{16–18}

Our purpose was to determine whether age-associated changes of aldosterone or mineralocorticoid receptor (MR) signaling dysregulation occur in vascular smooth muscle cells (VSMCs). Using aortic VSMCs isolated from Fisher 344 cross-bred Brown Norway (F344XBN) rats, we attempted to elucidate the possible effects of aldosterone and MR signaling on the mechanisms that underlie the arterial inflammation that accompanies arterial aging. The F344XBN rat model has been recommended in the 1990s as the preferred model for aging rat research. For example, the F344XBN rat shows longer maximal life span and normal distribution of age-related pathology and presents less age-related pathologies, such as renal dysfunction or cancer, than other rat strains. Thus, this model is considered to exhibit age-associated changes as a result of normal aging, rather than merely as a reflection of underlying disease. Furthermore, numerous studies over the last decade in this rat model have characterized an age-associated proinflammatory arterial phenotype.\textsuperscript{19,20}

As a first step, we compared MR expression in whole aortae and early passage VSMCs from adult (8 months) and aged (30 months) animals. We demonstrate that vascular MR is increased in aged animals, and sensitivity for aldosterone-induced extracellular signal-regulated kinase (ERK) 1/2 activation is enhanced in aged VSMCs. MR blockade by spironolactone or inhibition of the ERK1/2 mitogen-activated protein kinase (MAPK) pathway by UO126 prevents age-associated upregulation of proinflammatory gene expression, indicating involvement of MR signaling in the development of inflammation that is associated with arterial aging.

**Materials and Methods**

**Animals**

All of the procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee and
compiled with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 3040-2, revised 1999).

Eight- and 30-month-old Fisher344 X Brown Norway rats (F344XBN) were obtained from the National Institute on Aging Contract Colonies (Harlan Sprague Dawley, Indianapolis, IN). Animals were euthanized by an overdose of sodium pentobarbital, and thoracic aortae were processed immediately.

VSMC Isolation and Cell Culture
VSMCs were isolated enzymatically as described previously21,22 (please see the online Data Supplement at http://hyper.ahajournals.org for details).

Real-Time PCR Analysis
Total RNA was isolated using the RNeasy kit, according to the manufacturer’s instructions (Qiagen). Subsequently, RNA was reverse transcribed using random hexonucleotides for 30 minutes at 48°C, according to the manufacturer’s instructions (Applied Biosystems). Real-time PCR was performed using the SYBR Green protocol in a 384-well plate format, as described previously (Applied Biosystems)5 (please see the online Data Supplement for details).

Western Blot Analysis
Western blot analysis was performed as described previously23 (please see the online Data Supplement for details).

Quantification of ERK1/2 Phosphorylation by In Situ Cell-Based ELISA
For quantification of ERK1/2 phosphorylation we modified an in situ cell-based ELISA, described previously24,25 (please see the online Data Supplement for details).

Immunohistochemical Analysis
Immunohistochemical analysis was performed according to a slightly modified protocol applied by us earlier26 (please see the online Data Supplement for details).

Results
Aldosterone-induced vascular effects are mediated via MR and ERK1/2 MAPK–dependent pathways27 affecting transcription of proinflammatory genes promoting vascular inflammation.28–32 As shown in Figure 1A, MR mRNA is increased in whole aortic tissue from aged animals, as determined by real-time PCR. MR protein expression is enhanced in aortic VSMCs from aged rats. (Figure 1B and 1C). As shown in Figure 1D and 1E, angiotensin II (Ang II), but not aldosterone, increases MR protein in adult and aged VSMCs. We compared activation of ERK1/2 MAPK after stimulation with different aldosterone concentrations in VSMCs from adult and aged animals (Figure 2A). Aging shifts the aldosterone-induced ERK1/2 phosphorylation dose-response curve to the left and elevates maximal stimulation, whereas total ERK1/2 expression remains unchanged (Figure 2B), indicating increased sensitivity for aldosterone-mediated ERK1/2 activation in aged compared with adult VSMCs.

Cellular expression of TGF-β (2.1±0.3-fold), ICAM-1 (2.1±0.3-fold), and procollagen I (pro–Col-1; 1.8±0.2-fold) are significantly enhanced in untreated aortic VSMCs from aged compared with adult rats (Figure 3). MR blockade by spironolactone and inhibition of MAPK signaling by UO126 inhibit the age-associated increase of proinflammatory markers (Figure 3). Nanomolar aldosterone concentrations significantly enhanced cellular expression of TGF-β, ICAM-1, and pro–Col-1 in cells from adult animals, as demonstrated in Figure 4, indicating a shift of marker protein expression in adult cells toward a more aged expression pattern. However, no significant aldosterone-treatment effect could be observed in aged cells (please see Figure S2, available in the online Data Supplement at http://hyper.ahajournals.org).
Discussion

Arterial aging is associated with increased arterial renin-angiotensin system expression and activity accompanied by a proinflammatory phenotype that resembles early pathological changes during experimental induction of hypertension and atherosclerosis.\(^3\)\(^,\)\(^5\)\(^,\)\(^8\)\(^,\)\(^35\) Aldosterone can exert proinflammatory vascular effects via MR- and ERK1/2 MAPK-dependent mechanisms.\(^36\)

We demonstrate that MR mRNA in the arterial wall and protein levels in isolated VSMCs from F344XBN rats increase with age. Elevated Ang II expression and signaling are major characteristics of aged arteries,\(^57\)\(^,\)\(^38\) and Ang II is known to stimulate MR expression,\(^11\) hereby promoting MR-mediated expression of proinflammatory genes. Accordingly, Ang II stimulated MR expression in adult cells to a level resembling that of aged cells and further increased MR expression in aged VSMCs. However, whether Ang II truly is the major factor for increased MR expression in aged rat VSMCs and whether MR expression is elevated in healthy aged humans, as well as potential clinical implications, remain to be investigated in future studies. Moreover, aging...
shifts the aldosterone-induced ERK1/2 phosphorylation dose-response curve to the left and elevates maximal stimulation, indicating increased sensitivity for aldosterone-mediated MAPK activation in aged compared with adult VSMCs. In accordance with our hypothesis that increased MR expression and ERK1/2 MAPK activity are involved in the development of age-associated arterial inflammation, MR blockade by spironolactone and inhibition of MAPK signaling by UO126 reduced expression of proinflammatory markers in aged cells to a level resembling that of adult cells.

Nanomolar aldosterone concentrations increased proinflammatory marker expression in adult cells, indicating a shift toward a more aged phenotype. High (100 nmol/L) aldosterone concentrations may also activate glucocorticoid receptors, possibly opposing MR effects. In contrast, in aged cells aldosterone did not further stimulate proinflammatory marker expression, suggesting that MR is activated by ligand-independent means in aged cells and that no further activation is effected by aldosterone. Jaffe and Mendelsohn have shown that Ang II can directly activate the MR in coronary VSMCs, and local Ang II expression is known to be increased in aged arteries. MR activity can be modulated by a variety of coregulators. For example, rac-1 proved to be a potent activator of MR activity both in the presence and absence of aldosterone, and results from our previous studies indicate a moderate increase of rac-1 in aged arteries. Interestingly, in vitro MR overexpression in the absence of steroids also leads to ligand-independent ERK1/2 activation, supporting the

Figure 5. Aging and aldosterone both increase EGFR expression in VSMCs. EGFR expression is enhanced in untreated aortic VSMCs from aged compared with adult F344XBN rats, as demonstrated by Western blot (A) and immunofluorescence analysis (B). Nanomolar aldosterone concentrations (1 nmol/L) increase EGFR protein in (A) adult and (C) aged cells. Spironolactone (2 µmol/L) and UO126 (10 µmol/L) inhibit increased EGFR expression in aged cells (A). Blue indicates nuclear staining; green indicates fluorescein isothiocyanate secondary antibody staining of EGFR. Each blot is a representative blot shown above densitometric analysis. *P<0.05 vs adult control; §P<0.05 vs aged control; n.s. indicates nonsignificant.

Figure 6. Inhibition of EGFR tyrosine kinase reduces age-associated inflammatory marker expression. Aged cells were treated with EGFR tyrosine kinase inhibitor AG1478 (200 nmol/L) for 48 hours. AG 1478 reduces expression of (A) TGF-β, (B) ICAM-1, and (C) pro–Col-1 in aged cells. Each blot is a representative blot shown above densitometric analysis. *P<0.05 vs adult control; §P<0.05 vs aged control.
hypothesis that increased proinflammatory marker expression in aged cells, other than possible other mechanisms, might be attributed to ligand-independent MR and ERK1/2 activation. However, this reflects an untested hypothesis, and future research is needed to explore in detail the mechanisms that might account for increased MR activity in aged arteries in vivo.

Aldosterone effects involving transcriptional activity of MR are referred to as classic or genomic ways of action. Rapid aldosterone effects, also referred to as nongenomic effects, are typically observed within minutes after aldosterone stimulation and include activation of MAPK p38, Jun N-terminal kinase, or ERK1/2. Aldosterone-mediated pathologies, such as inflammation, remodeling, and endothelial dysfunction, are characterized by activation and cross-talk of genomic effects with rapid signaling pathways. According to this view, aldosterone-induced activation of MAPKs can modulate the activity and cellular expression pattern of various proteins. For example, in VSMCs aldosterone activates NADPH oxidase in an ERK1/2-dependent way, leading to the production of chemokines and cytokines promoting vascular inflammation. The present study examines the expression of TGF-β, ICAM-1, and pro–Col-1 after 48 hours of aldosterone stimulation. Thus, our results cannot discriminate to what extent genomic, nongenomic, and secondary compensatory mechanisms eventually contribute to the observed effects. Thus, MR-mediated genomic actions or MR-mediated nongenomic MAPK signaling may both play a significant role in age-associated proinflammatory marker expression in our cell model.

Cross-talk of rapid aldosterone signaling with the EGFR or Ang II pathways has been described. Aldosterone has been shown to enhance Ang II–mediated ERK1/2 activation in VSMCs and to increase vascular angiotensin-converting enzyme expression, local Ang II concentrations, and Ang II type 1 receptor expression. EGFR is a key factor promoting vascular damage, and transactivation of EGFR is involved in the mediation of vascular Ang II, endothelin 1, and catecholamine effects, all of which have been linked to hypertension, vascular inflammation, and arteriosclerosis. Age-associated upregulation of EGFR in the arterial wall increases sensitivity for ERK1/2 MAPK activation, eventually enhancing the deleterious effects of Ang II, endothelin 1, or aldosterone. Both MR and c-src–mediated transactivation of the EGFR mediate aldosterone-induced ERK1/2 activation in VSMCs, leading to increased oxidative stress and inflammation. Our study shows that EGFR expression is stimulated by low aldosterone (1 nmol/L) in adult and aged VSMCs and increases with age, suggesting that aldosterone might indirectly mediate adverse effects in the aging arterial wall by increasing EGFR expression. Accordingly, inhibition of EGFR kinase in aged cells reduced expression of TGF-β, ICAM-1, and pro–Col-1 to levels resembling those of the adult cell. Ang II did not elevate EGFR expression (data not shown). Thus, enhanced MR and EGFR expression and signaling may contribute to inflammation in aging arteries.

Vascular aldosterone production and possible age-associated changes might crucially affect arterial aging. However, the evidence for local aldosterone production in the vasculature or the heart is contradictory. A study by Takeda et al demonstrated aldosterone production from mesenteric arteries of Wistar-Kyoto rats and human endothelial cells. In contrast, results from Ahmad et al do not support the former results, challenging vascular aldosterone production. To date it is not known whether aldosterone is produced at a considerable amount in the vessel wall and whether it increases with age.

Our study focused on cell-specific, aging-related vascular alterations in aldosterone/MR signaling. Using VSMCs from adult and aged F344×BN rats, we were able to avoid unknown influences and interactions with other cell types, such as endothelial cells, in animal models. Our results provide in vitro evidence supporting the hypothesis that increased constitutive MR signaling may contribute to age-associated inflammation that accompanies arterial aging through increased Ang II–stimulated expression of MR and enhanced sensitivity to aldosterone-mediated ERK1/2 activation, likely related to increased EGFR expression.

Perspectives
Aging is considered the major cardiovascular risk factor. Central arterial aging is characterized by a proinflammatory phenotype leading to arterial remodeling with wall thickening and stiffening. These age-associated changes render the arterial wall a fertile substrate for age-associated diseases, such as hypertension and atherosclerosis, indicating that aging and diseases are fundamentally intertwined at the cell and molecular levels. The nature of these age-disease interactions is very complex, including well-defined external risk factors, such as NaCl consumption, genetic factors, and mechanisms of aging. This concept calls for new kinds of interventional (preventional) strategies fighting subclinical functional and structural arterial changes in health to reduce the incidence and prevalence of cardiovascular diseases. Chronic pharmacological angiotensin-converting enzyme inhibition or Ang II type 1 receptor blockade prevent the onset and progression of age-associated arterial remodeling in animal models. However, it is thus far unproven whether these interventions can slow down age-associated arterial remodeling in healthy individuals who exhibit significant subclinical evidence of “unsuccessful aging.” This report provides evidence for a role of MR and aldosterone signaling in the age-associated inflammation that accompanies arterial aging, expanding the spectrum of factors that might orchestrate arterial aging in vivo. Our in vitro results encourage future studies aiming to further characterize the biological relevance of MR and aldosterone signaling in arterial aging in vivo and possibly paving the way into a new field of MR blocker application as a preventive treatment for cardiovascular diseases.

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TITLE: ELEVATED MR ACTIVITY IN AGED RAT VSMCs PROMOTES A PROINFLAMMATORY PHENOTYPE VIA ERK1/2 MAPK AND EGFR-DEPENDENT PATHWAYS


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DATAILED MATERIALS AND METHODS:

Vascular smooth muscle cell (VSMC) isolation and cell culture:

VSMCs were isolated enzymatically. In short, rat thoracic aortae were rinsed in Hanks’ balanced salt solution containing 50g/ml streptomycin, 50g/ml penicillin, and 0.25g/ml amphotericin B (Life Technologies). After digestion for 30 minutes in 2 mg/ml of collagenase I solution (Worthington Biomedical) at 37°C, the adventitia and intima were removed from the vessel media layer. The latter was placed in complete cell culture medium (DMEM plus 10% fetal calf serum, Invitrogen) overnight. On the following day, the arterial media was further digested with 2 mg/ml of collagenase II-0.5 mg/ml elastase (Sigma) for 1 hour at 37°C. Subsequently, the isolated cells were washed and plated in complete medium. Cell phenotype was verified by staining for α-smooth muscle actin (α-SMA), which was positive in > 95% of cells. The culture
medium was changed every 2 days, until cells had reached approximately 80% confluence. Prior to experiments, cells were left in serum-free medium for 12-16 h, and subsequently stimulated with aldosterone, angiotensin II or inhibitors for 48h, and this was followed by protein or mRNA isolation.

**Experimental and statistical approach**

For PCR analysis, aortae from 4 different animals were isolated. Cells from each individual animal were cultured separately, cells were not pooled. Each sample was analyzed in quadruplicates. The mean of the quadruplicates is considered n=1. This procedure was repeated with 4 different aortae. Therefore, n represents the number of animals from which cells were derived for each experimental series, and the total number of animals n = 4.

For Western blot analysis and ELISA experiments, aortae from 5-8 different animals were isolated. Cells from each individual animal were cultured separately, cells were not pooled.

For Western blot analysis, cells from one animal were plated in 6-well plates and 3 wells were used for each experimental treatment. Protein from each well was isolated and processed separately; thus, from each experimental treatment 3 separate samples (triplicates) were available for Western blotting. Each of these samples was used separately for Western blot analysis, ODs were generated and the mean from these three ODs is considered n=1. This procedure was repeated with 5-8 different aortae. Therefore, n represents the number of animals from which cells were derived for each experimental series, and the total number of animals n = 5-8. All data were normalized to beta actin.

For ELISA analysis, cells were plated in 96-well plates and 3 wells were used for each experimental treatment (triplicates). The mean of these 3 wells is considered n=1. This procedure was performed with 5-8 aortae, each cultured separately. Therefore, n represents the number of animals from which cells were derived for each experimental series, and the total number n = 5-8. All data were normalized to cell content/well (as described under the section Quantification of ERK1/2 phosphorylation).

Normality was assessed by using the Shapiro-Wilk test, in addition to the normal probability plots and frequency histograms, and Equal Variance tests were performed. This was followed by testing for age and treatment and their interaction by two-way analysis of variance (ANOVA). One-way ANOVA was performed in case of comparison of multiple different treatments only – without comparison for age. Post-hoc comparisons were further performed to test the levels of treatment (Bonferroni t-test). t-test was applied when comparing two different groups only. Differences were considered significant if \( p \) was <0.05.

Original data is used for the ANOVA analysis but represented using percent control (100%) data for better visualization.

**Real-Time PCR Analysis:**
The PCR reaction conditions were: 10 min at 95°C (one cycle), and 15 sec at 95°C, 20 sec at 60°C, 30 sec at 72°C (40 cycles). Gene-specific PCR products were measured using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The following primers were used for specific amplification: Each sample was tested in quadruplicate. Samples were normalized to GAPDH expression. Data are expressed as mean using the following formula: quantity=10^{-((Ct-Y \text{ intercept/slope value})/2)}. Ct represents the threshold cycle value\(^3\). We used the following primers for MR detection:

Forward primer: TGATTGACAGTTGGTGGCA
Reverse primer: TGGGAGTGAGAGGGAAGAGT

Western blot analysis:

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold Cytobuster Protein Extraction Reagent (Novagen) containing 1% protease inhibitor cocktail (Sigma). Cell lysates were matched for protein content, separated by SDS-PAGE (Invitrogen) and transferred to PVDF membrane (Immobilon). After blocking, the membrane was incubated with primary rabbit anti-osteopontin antibody (AB1870, Chemicon), rabbit anti-MR (sc-11412, Santa Cruz Biotechnology), rabbit anti-11beta HSD-2 (BHSD21-A, Alpha Diagnostics), rabbit anti-c-src (sc-19, Santa Cruz Biotechnology), rabbit anti EGFR (sc-03, Santa Cruz Biotechnology), mouse anti-TGF-beta (MAB1032, Chemicon), mouse anti-ICAM-1 (MAB2146, Chemicon), rabbit anti-p44/42 MAPKinase (9102, Cell Signalling), rabbit anti-procollagen-1 (Rockland). Bound primary antibody was detected using the Western Breeze Detection Kit (Invitrogen) and the SuperSignal West Pico Chemiluminescence substrate (Pierce Technology). Densitometric analysis was performed using ImageJ software and OD was normalized to ß-actin\(^4\). Only cells from the same cell passage, same experiment and the same western blot membrane were compared with each other.

Quality of anti-MR was evaluated using hMR-EGFP-transfected human embryonic kidney (HEK) cells. Supplementary figure 1 shows bands of the same molecular weight of approximately 140kD (MR+ 30kD EGFP) applying either anti-EGFP or anti-MR as primary antibodies using lysates of hMR-EGFP transfected cells. This demonstrates the specificity of the used antibody for MR as (a) mock-transfected cells do not exhibit reactivity against this antibody and (b) anti-EGFP antibody detects a band of the same molecular weight as anti-MR antibody.

Quantification of ERK1/2 phosphorylation by in situ cell-based ELISA:

For quantification of ERK1/2 phosphorylation we modified an in situ cell-based ELISA. Cells were seeded in 96 well plates at a density of 5,000 cells/0.32 cm\(^2\). After reaching approximately 80% confluence they were serum-starved for 12-16 h. Subsequently, cells were equilibrated in 1x Hepes-Ringer solution (130.0 mmol/liter NaCl, 5.4 mmol/liter KCl, 1.0 mmol/liter CaCl\(_2\), 1.0 MgCl\(_2\), 1.0 mmol/liter NaH\(_2\)PO\(_4\), 10 mmol/liter HEPES, and 5 mmol/liter glucose (pH 7.4) at 37 °C for 30 min and then stimulated in the same buffer plus the respective vehicle (DMSO) with or without
aldosterone (0.1-100 nmol/l). Immediately afterwards, cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature and washed three times for 5 min with 0.1% Triton-X 100 in PBS. Endogenous peroxidase activity was quenched for 20 min with freshly prepared 1% H$_2$O$_2$ and 0.1% azide. Cells were washed again three times in the same buffer, blocked with 5% bovine serum albumin (BSA) in PBS/Triton for 1h and finally incubated overnight with the primary antibody (rabbit anti-p44/42 MAPKinase, Cell Signaling) in PBS/Triton containing 5% BSA at 4°C under gentle shaking. The next day, cells were washed three times with PBS/Triton and incubated with the HRP-coupled secondary antibody (1:5000) in PBS/Triton containing 5% BSA for 1h at room temperature, and afterwards washed three times with PBS/Triton for 5 min and 10 min with PBS. Cells were then incubated in 100 µl of TMB substrate solution (eBioscience) for 20 min in the dark. The resulting signals were read using a multiwell reader (Victor$^2$, Wallac). Subsequently, cells were washed twice with PBS/Triton and twice with PBS. The wells were air-dried for 5 min at room temperature and then stained with 100 µl of trypan blue solution (0.2% in PBS) for 60 min at room temperature. Cells were then washed for three times with PBS and demineralized water, then 100 µl of 1% SDS solution was added and the plate was incubated on a shaker for 1h at room temperature. Finally, the absorbance was measured at 595 nm. For each well, 490 nm signals (ERK1/2 phosphorylation) were divided by 595 nm readings (trypan blue method), allowing to normalize ERK1/2 phosphorylation for total protein content in each well$^5$.

**Immunohistochemical analysis:**

VSMCs were seeded onto collagen-coated glass coverslips and after reaching approximately 80% confluence, they were incubated with 100 nmol/l aldosterone for 48h. Fixation was performed in 4% paraformaldehyde/PBS at room temperature for 20 min. After three times washing in PBS, cells were blocked using blocking solution for 30min at room temperature, followed by incubation with primary antibodies (rabbit anti-EGFR, Santa Cruz Biotechnology; rabbit anti-MR, Santa Cruz Biotechnology) in antibody diluent at 4°C overnight. After washing three times in PBS, cells were incubated with FITC-conjugated secondary antibodies in antibody diluent (Invitrogen) (1:500) at room temperature for 60min followed by washing in PBS. Nuclear counterstaining was performed using TOPRO-3 dye (Invitrogen). Coverslips were then mounted in Vectashield Mounting Media (Vector Laboratories)$^6$. Confocal microscopy was performed with a Zeiss-LSM19 confocal microscope with a 60X objective lens.

**References**


Supplementary figure S1:
Quality of anti-MR was evaluated using hMR-EGFP-transfected human embryonic kidney (HEK) cells. Supplementary figure 1 shows bands of the same molecular weight of approximately 140kD (MR+ 30kD EGFP) applying either anti-EGFP or anti-MR as primary antibodies using lysates of hMR-EGFP transfected cells. This demonstrates the specificity of the used antibody for MR as (a) mock-transfected cells do not exhibit reactivity against this antibody and (b) anti-EGFP antibody detects a band of the same molecular weight as anti-MR antibody.
Supplementary Figure S2 a

TGF-β

β-actin

relative TGF-β expression (% of aged)

n.s.  n.s.  n.s.

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<th>Con</th>
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+ Aldosterone (nmol/l)
Supplementary figure S2 b

ICAM-1

β-actin

relative ICAM-1 expression (% of aged)

n.s.  n.s.  n.s.

Con  1  10  100

+ Aldosterone (nmol/l)
Supplementary figure S2 c: Aldosterone does not increase proinflammatory marker proteins in aged. TGF-β (a), ICAM-1 (b) and pro-collagen-1 (c) expression in VSMCs from aortae of aged F344XBN rats w/wo 48h aldosterone stimulation (1-100 nmol/l). No significant differences were observed between control and aldosterone-treated cells in aged animals (n.s. non significant, p>0.05). Each blot is a representative blot shown above densitometric analysis.
Supplementary figure S3: Aging does not change c-src expression in VSMCs. C-src expression is unchanged in untreated aortic VSMCs from aged compared to adult F344XBN rats, as demonstrated by Western Blot. Each blot is a representative blot shown above densitometric analysis. n.s. non significant.