Angiotensin II–aldosterone interaction in human coronary microarteries involves GPR30, EGFR, and endothelial NO synthase

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Aims
The aim of this study was to investigate the aldosterone–angiotensin (Ang) II interaction in human coronary microarteries (HCMAs).

Methods and results
HCMAs, obtained from 75 heart-beating organ donors, were mounted in myographs and exposed to Ang II, either directly or following a 30-min pre-incubation with aldosterone, 17β-oestradiol, hydrocortisone, the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, the extracellular regulated kinase 1/2 (ERK1/2) inhibitor PD98059, the GPR30 antagonist G15, or the epidermal growth factor receptor (EGFR) antagonist AG1478. Ang II constricted HCMAs in a concentration-dependent manner. All steroids, at nanomolar levels, potentiated Ang II and G15 prevented this effect. The potentiation disappeared or was reversed into Ang II antagonism at micromolar steroid levels. NO synthase (NOS) inhibition prevented the latter antagonism in the case of 17β-oestradiol, whereas both aldosterone and 17β-oestradiol induced endothelial NOS phosphorylation in human umbilical vein endothelial cells. AG1478, but not SB203580 or PD98059, abolished the Ang II-induced contraction in the presence of aldosterone or 17β-oestradiol, and none of these drugs affected Ang II alone.

Conclusion
Steroids including aldosterone affect Ang II-induced vasoconstriction in a biphasic manner. Potentiation occurs at nanomolar steroid levels and depends on GPR30 and EGFR transactivation. At micromolar steroid levels, this potentiation either disappears (aldosterone and hydrocortisone) or is reversed into an inhibition (17β-oestradiol), and this is due to the endothelial NOS activation that occurs at such concentrations.

1. Introduction
Aldosterone antagonism on top of renin–angiotensin system (RAS) blockade reduces mortality in patients with heart failure. Since this could not be attributed to blockade of renal mineralocorticoid receptors (MRs), it is now generally accepted that the reduced mortality with MR blockade is due to interference with aldosterone’s extrarenal effects, particularly, in the heart. Indeed, cardiomyocyte-specific MR overexpression in mice results in coronary endothelial dysfunction. Moreover, MRs do occur in the human heart, and are up-regulated under pathophysiological conditions. Importantly, the cardiac expression of the cortisol-degrading enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) is increased in patients with cardiomyopathy. Without this enzyme, cortisol, given its 1000-fold higher levels in extracellular fluid and equal affinity for the MR, would act as the endogenous agonist of the cardiac MR. However, with this enzyme, like in the kidney, aldosterone may act as the endogenous agonist of the MR in the heart. In humans, aldosterone affects cardiac inotropy, causes both dilatory and constrictor effects, and induces plasminogen activator inhibitor-1 release. Its dilatory effects are mediated via NO and depend on the activation of endothelial NO synthase (eNOS) via the phosphatidylinositol 3-kinase (PI3-K) pathway. Aldosterone potentiates angiotensin (Ang) II, resulting not only in an enhanced vasoconstriction in human coronary arteries, but also in increased proliferation, migration, and senescence of...
rat vascular smooth muscle cells. The underlying mechanism(s), derived from animal studies, involve modulation of Ang II type 1 (AT₁) receptor expression, AT₁ dimer formation, transactivation of the epidermal growth factor (EGFR), and/or activation of the epithelial sodium channel. The intracellular pathways that are thus augmented, are the extracellular regulated kinase 1/2 (ERK1/2) and c-Jun NH₂-terminal protein kinase cascades, and c-Src-dependent activation of NAD(P)H oxidase. Although aldosterone also activates p38 mitogen-activated protein kinase (MAPK), this pathway is not necessarily involved in the combined Ang II/aldosterone effect. To what degree the Ang II–aldosterone interaction involves the MR remains unclear, since MR antagonists could not always block it. In support of a role for MR, endothelial cell-specific MR overexpression per se is sufficient to develop hypertension, and mesenteric arteries of such transgenic mice display enhanced contractile responses to constrictor agents, including Ang II. Recently, however, it has been suggested that the acute effects of aldosterone might be mediated via the G protein-coupled receptor GPR30, a cell surface receptor that is also activated by 17b-oestradiol.

Given the scarcity of human data on Ang II–aldosterone interaction, in the present study, we set out to investigate such interaction in human coronary microarteries (HCMAs), focusing on the contribution of ERK1/2, p38 MAPK, NO, GPR30, and the EGFR. In view of the combined dilator and constrictor effects of aldosterone in humans, we hypothesized that aldosterone may both antagonize and potentiate Ang II, most likely in a concentration-dependent manner. We, therefore, tested a wide range of aldosterone concentrations, and made a comparison with other steroids (17b-oestradiol and hydrocortisone) that are known to oppose or potentiate Ang II via the same receptors (MR, GPR30). Finally, making use of human endothelial cells, we verified the concentration-dependency of steroid-induced eNOS activation.

2. Methods

2.1 Tissue collection

HCMAs were obtained from 75 heart-beating organ donors (35 males, 40 females, age 11–66 years, mean 47 years), who died of non-cardiac causes (17 cerebrovascular accident, 24 head trauma, 23 subarachnoid bleeding, 7 post-anoxic encephalopathy, 4 intracranial bleeding) <24 h before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves for transplantation purposes. The study was approved by the Ethics Committee of the Erasmus MC, and studies occurred conform the principles outlined in the Declaration of Helsinki. The hearts were stored in an ice-cold sterile organ-protecting solution after circulatory arrest. After arrival at the laboratory, a tertiary branch of the left anterior descending coronary artery (inner diameter 260–600 μm, mean 420 μm) was removed and stored overnight in a cold (4 °C), oxygenated Krebs bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 8.3; pH 7.4.

Porcine renal arteries (PRAs) were removed from kidneys obtained from the local slaughterhouse. PRAs were used, rather than porcine coronary arteries, because the latter virtually do not respond to Ang II, whereas PRAs do. Vessels were stored overnight as described above. Storage in oxygenated Krebs bicarbonate solution did not affect vascular function, and vessels retained excellent endothelium-dependent relaxation.

2.2 Myograph studies

Following overnight storage, HCMAs were cut into segments of ~2 mm length and mounted in a Mulvany myograph (J.P. Trading, Aarhus, Denmark) with separated 6-mL organ baths containing Krebs bicarbonate solution, aerated with 95% O₂ and 5% CO₂, and maintained at 37 °C. Tissue responses were measured as changes in isometric force, using a Harvard isometric transducer. Following a 30-min stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure as described by Mulvany and Halpern. Endothelial integrity was verified by observing relaxation to 10 nmoL/L substance P after pre-constriction with 10 nmoL/L of the thromboxane A₂ analogue U46619. Relaxation was always better than 90%. Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 nmoL/L KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min. Next, segments were pre-incubated for 30 min with aldosterone, 17b-oestradiol, hydrocortisone (1 nmoL/L–10 μmoL/L for all three steroids), the ERK1/2 inhibitor PD98059 (10 μmoL/L), the p38 MAPK inhibitor SB203580 (10 μmoL/L), the EGFR antagonist AG1478 (10 μmoL/L), the glucocorticoid receptor (GR) antagonist RU486 (mifepristone; 1 μmoL/L), the MR antagonist eplerenone (1 μmoL/L), the GPR30 antagonist G15 (1 μmoL/L), and/or the NOS inhibitor Nω-nitro-L-arginine methyl ester HCl (L-NAME; 100 μmoL/L). Thereafter, concentration–response curves (CRCs) were constructed to Ang II. The cyclo-oxygenase inhibitor indomethacin (5 μmoL/L) was present during the entire experiment to suppress spontaneously occurring contractions and relaxations. All drugs were from Sigma-Aldrich (Zwijndrecht, The Netherlands).

PRAs were cut into segments of ~4–5 mm and mounted in organ baths as described before. The resting tension was set at 2 g. After verifying endothelial integrity, vessels were pre-constricted with 0.3 μmoL/L phenylephrine or 0.1 μmoL/L Ang II and exposed to aldosterone (1 nmoL/L–10 μmoL/L).

2.3 Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords, cultured to confluence, trypsinized, and stored in liquid nitrogen as described previously. For a typical experiment, an aliquot of HUVECs (passage 2 to 5) was thawed. The cells were seeded in a 75 cm² tissue culture flask coated with human fibronectin (10 μg/cm²) and cultured to confluence, trypsinized, and stored in liquid nitrogen as described previously. For a typical experiment, an aliquot of HUVECs was used. HUVECs were seeded in 75 cm² tissue culture flask coated with human fibronectin (10 μg/cm²) and cultured to confluence in supplemented DMEM medium (Invitrogen, Grand Island, NY, USA) containing 20% newborn calf serum and 2% human serum. The cells were trypsinized, and seeded into 6-well plates using the above medium. This yielded a confluent monolayer of ~4 × 10⁵ cells/cm² after 3 days. Before the start of the experiment, the cells were cultured for 24 h under serum-free conditions in DMEM medium.

2.4 Quantification of endothelial NO synthase (eNOS) phosphorylation, MR, and GPR30

HUVECs were incubated with 17b-oestradiol or aldosterone (0.01 or 10 μmoL/L) for 15 min. Cells incubated with bradykinin (1 μmoL/L) for 15 min served as positive control. After this period, the cells were lysed using Nonidet P-40 (NP40) lysis buffer, and kept on ice for 15 min. Next, the cell lysates were centrifuged at 14000 g for 20 min at 4 °C. Supernatants were collected and stored at −20 °C until further analysis.

Western blotting was performed with 15 μg of protein using total eNOS antibody and phospho-eNOS (ser1177) antibody (both diluted 1:1000; Cell Signaling Technology, Danvers, MA, USA). Frozen samples of left ventricular (LV) tissue and HCMAs from 6 human hearts were homogenized in NP40 lysis buffer, and kept on ice for 1 h. After this period, the lysates were treated as described above. Western blotting for MR and GPR30 protein expression was performed with 25 μg of protein using MR antibody H-300 and GPR30 antibody K-19-R (both diluted 1:500; Santa Cruz, Santa Cruz, CA, USA). To verify loading of
the samples, an antibody against β-actin (C4) was used (diluted 1:50,000, Santa Cruz). Protein of all samples was determined using the BCA (bicinchoninic acid) protein assay (Thermo Scientific, Etten-Leur, The Netherlands). A peroxidase-conjugated antibody (goat anti-mouse in the case of β-actin and goat anti-rabbit for all others, 1:5000) was used to visualize total and phosphorylated eNOS, MR, GPR30, and β-actin.

2.5 Data analysis
Data are given as mean ± SEM. Contractile responses are expressed as a percentage of the contraction to 100 mmol/L K+. CRCs were analysed as described before to obtain pEC50 (−10logEC50) and maximum effect (Emax) values.37 Endothelial NOS phosphorylation was expressed as a percentage of baseline values relative to total eNOS. MR and GPR30 protein expression was expressed in arbitrary units (a.u.) relative to β-actin expression in each sample. Statistical analysis was by the paired t-test or one-way ANOVA, followed by Dunnett’s post hoc evaluation. P < 0.05 was considered significant.

3. Results

3.1 Myograph studies
Ang II constricted HCMAs in a concentration-dependent manner (pEC50 8.4 ± 0.1, n = 67) with a maximal response (Emax) of 58 ± 4.5%. Administration of aldosterone (1 nmol/L−10 μmol/L) on top of Ang II did not exert constrictor or dilator effects within 5–10 min (response in the presence of the highest aldosterone concentration 98 ± 1% of the response without aldosterone, n = 3), nor did aldosterone relax phenylephrine or Ang II-pre-constricted PRAs (response in the presence of the highest aldosterone concentration 101 ± 5 and 112 ± 6%, respectively, of the response without aldosterone, n = 3). Pre-incubation with aldosterone, 17β-oestradiol, and hydrocortisone, at concentrations ranging from 1 nmol/L to 10 μmol/L, did not affect basal vascular tone. Pre-incubation with aldosterone at a concentration of 1 nmol/L (n = 22) did not affect the Ang II CRC (Figure 1). However, pre-incubation with aldosterone, at a concentration of 10 nmol/L (n = 15), shifted the Ang II curve to the left by about two-fold (pEC50 8.6 ± 0.1; P < 0.05) and increased Emax (81 ± 7.3 vs. 59 ± 10%; P < 0.05). Following pre-incubation with aldosterone at higher concentrations (n = 9–23), its potentiating effects disappeared.

A comparable pattern was observed following pre-incubation with 17β-oestradiol (Figure 2). At concentrations of 1 nmol/L (n = 13) and 10 nmol/L (n = 5), it increased the maximum effect of Ang II to 92 ± 13 and 115 ± 23%, respectively (P < 0.01 for both). At higher 17β-oestradiol concentrations, this potentiating effect disappeared (n = 6–12), until at a concentration of 10 μmol/L 17β-oestradiol, the effect of Ang II was found to be virtually abolished (Emax 5.3 ± 2.4%, n = 10; P < 0.001). Under no condition did pre-incubation with 17β-oestradiol affect the pEC50 of Ang II.

Finally, identical trends were observed for pre-incubations with hydrocortisone (Figure 3), in that a potentiating effect (higher Emax) towards Ang II was observed at nanomolar hydrocortisone concentrations, which disappeared at micromolar concentrations of the steroid. However, none of these differences was significant (n = 5–6).

The GPR30 antagonist G15 abolished the potentiating effects of both 10 nmol/L aldosterone and 1 nmol/L 17β-oestradiol (n = 7 for each, Figure 4A and B; steroid concentrations are the lowest concentrations observed in Figures 1 and 2 that induce potentiation), indicating that GPR30 is responsible for this phenomenon. To investigate whether the disappearance of the aldosterone-induced potentiation at higher aldosterone concentrations involved stimulation of the GR and/or MR, Ang II CRCs were constructed following pre-incubation with 1 μmol/L aldosterone in the presence or absence of eplerenone with or without RU486. However, neither eplerenone nor eplerenone + RU486 (n = 5–6; Figure 4C) altered the Ang II CRC in the presence of 1 μmol/L aldosterone.

The ERK1/2 inhibitor PD98059, the MAPK p38 inhibitor SB233580 and the EGFR antagonist AG1478 (10 μmol/L) did not alter the response to Ang II alone (n = 7–18; Figure 5, left panel). However, 10 μmol/L AG1478 (Figure 5, middle and right panels), fully blocked the effect of Ang II in the presence of 1 μmol/L aldosterone (Emax 4.8 ± 4%; n = 4; P < 0.05) or 1 μmol/L 17β-oestradiol (Emax 3.1 ± 3%; n = 4; P < 0.05). A 100-fold lower concentration of AG14178 exerted no effect (data not shown, n = 3), nor did any of the other inhibitors interfere with the Ang II response in the presence of aldosterone (Figure 5, middle panel). These data indicate that the Ang II-steroid interaction resulting in constriction depends on the EGFR.

To evaluate whether the disappearance of the Ang II-potentiating effects of aldosterone and 17β-oestradiol at micromolar...
concentrations was due to the stimulation of eNOS at these concentrations, we also studied the effect of pre-incubation with aldosterone or 17β-oestradiol in the presence of the NOS inhibitor L-NAME. L-NAME did not alter the response to Ang II (n = 9, data not shown). It greatly increased the response to Ang II in the presence of 10 μmol/L 17β-oestradiol (E$_{max}$ 32 ± 10 vs. 7.5 ± 5.7%, respectively, n = 4–5; P < 0.05), but not in the presence of 1 μmol/L aldosterone (n = 7–14; P = NS) (Figure 6A).

### 3.2 Expression of MR and GPR30 in HMCAs and left ventricular tissue

Both GPR30 and MR protein were detected in LV tissue and HMCAs, and corrected for β-actin content, LV tissue showed the highest expression of both receptors (Figure 4D).

### 3.3 Phosphorylation of eNOS

Phosphorylated eNOS levels in HCMA segments, even when pooled, remained below the detection limit of our assay under all conditions (n = 3, data not shown), thus not allowing us to study this phenomenon in human coronary arteries. Steroid-induced eNOS phosphorylation was therefore further investigated in HUVECs. Bradykinin increased eNOS phosphorylation in HUVECs by 167 ± 52% (n = 6; P < 0.05). Similar increases were observed with aldosterone and 17β-oestradiol at a concentration of 10 μmol/L (n = 6; P < 0.05 for both), but not at a concentration of 10 nmol/L (n = 6; P = NS) (Figure 6B).

### 4. Discussion

The present study shows that pre-incubation with aldosterone, at a concentration of 10 nmol/L, enhances the contractile effects of Ang II in HMCAs in a rapid, ‘non-genomic’ manner via GPR30 activation. The latter observation explains why, in earlier studies, it was not possible to block this potentiation with MR antagonists such as spironolactone, eplerenone, and canrenone. At higher (micromolar) concentrations of aldosterone, the Ang II potentiation was no longer seen, most likely because aldosterone, at such concentrations, also
Figure 4 (A–C) Contractions of HCMAs to angiotensin II in the absence or presence of aldosterone [aldo; 10 nmol/L (A) or 1 μmol/L (C)] or 17β-oestradiol [oestr; 1 nmol/L (B)] with or without G15 (1 μmol/L), eplerenone (epl; 1 μmol/L), or RU486 (1 μmol/L). Contractions (mean ± SEM, n = 7) have been expressed as a percentage of the response to 100 mmol/L K⁺. (D) Protein expression of GPR30 (38 kDa, left panel) and MRs (102 kDa, right panel) in left ventricular (LV) tissue and HCMAs. Data (n = 6) are expressed as arbitrary units relative to β-actin (43 kDa) expression in each sample. Insert, respective expression of GPR30, MRs and β-actin in LV tissue and HCMAs. Please note that the first four samples were processed within 1 month after freezing, whereas the last two had been stored for almost 1 year.

Figure 5 Contractions of HCMAs to angiotensin II in the absence (A, no incubation) or presence of aldosterone (B, 1 μmol/L), or 17β-oestradiol (C, 1 μmol/L) following a pre-incubation with 10 μmol/L AG1478, 10 μmol/L PD98059 or 10 μmol/L SB203580. Contractions (mean ± SEM, n = 7–18) have been expressed as a percentage of the response to 100 mmol/L K⁺. *p < 0.01 vs. 

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Figure 5 Contractions of HCMAs to angiotensin II in the absence (A, no incubation) or presence of aldosterone (B, 1 μmol/L), or 17β-oestradiol (C, 1 μmol/L) following a pre-incubation with 10 μmol/L AG1478, 10 μmol/L PD98059 or 10 μmol/L SB203580. Contractions (mean ± SEM, n = 7–18) have been expressed as a percentage of the response to 100 mmol/L K⁺. *p < 0.01 vs. 

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induced eNOS phosphorylation, thereby counteracting its constrictor effects. A similar pattern was observed for 17β-oestradiol, and the 17β-oestradiol-induced Ang II potentiation also depended on GPR30 activation. The contraction-potentiating effects of 17β-oestradiol at nanomolar concentrations did not only disappear at higher (micromolar) concentrations, but were then in fact reversed into a relaxant effect, thereby annihilating the contractile response to Ang II. The fact that L-NAME was able to block the relaxant effect of 17β-oestradiol in HCMAs, combined with the 17β-oestradiol-induced eNOS phosphorylation at micromolar (but not nanomolar) concentrations in HUVECs, strongly suggests that 17β-oestradiol at micromolar concentrations induces NO release. Most likely, the NO release induced by 17β-oestradiol is larger than that induced by aldosterone, so that only in the presence of 17β-oestradiol the Ang II-induced vasoconstriction disappeared. Indeed, we were unable to show aldosterone-induced relaxant effects, both in Ang II-pre-constricted HCMAs and in phenylephrine or Ang II-pre-constricted PRAs. Nevertheless, in vivo, in humans, NO-dependent relaxant effects to aldosterone have been observed, demonstrating that, depending on vascular bed and/or experimental conditions, significant aldosterone-induced NO release may occur.

Rapid, non-genomic 17β-oestradiol-induced NO release is known to involve eNOS activation by oestrogen receptor α via the PI3-K-Akt pathway, although a role for oestrogen receptor β and GPR30 cannot be totally excluded. NO causes vasorelaxation via the soluble guanylyl cyclase-cGMP pathway, thus counteracting Ang II through physiological antagonism, and/or it decreases the binding affinity of the AT1 receptor by S-nitrosylation of cysteine 289 of the receptor.

Observations with hydrocortisone were similar to those with aldosterone, although no significant increases in Ang II response occurred at any hydrocortisone concentration, possibly due to the low n-number in these studies and/or because hydrocortisone is only a weak agonist of GPR30. Glucocorticoid-induced Ang II potentiation has been observed before, and may indeed involve activation of MR/GPR30 rather than GRs. The reverse effect, i.e. aldosterone exerting its effects via GR rather than MR, is also possible, at least when applying micromolar aldosterone concentrations (which are required to stimulate GR). Such GR activation might underlie the disappearance of the GPR30-mediated Ang II potentiation. If so, one would expect a return of the aldosterone-induced Ang II potentiation at micromolar aldosterone concentrations in the presence of the GR antagonist RU486. However, RU486 (1 μmol/L) did not alter the Ang II-induced responses in the presence of 1 μmol/L aldosterone. Similarly, in an earlier study in the human heart, RU486 did not block the effects of micromolar aldosterone concentrations. Thus, we were unable to demonstrate GR-mediated effects by aldosterone in the human heart, and GR activation does not counteract the GPR30-mediated effects.

A second possibility is that simultaneous MR activation counteracts the GPR30-mediated Ang II potentiation, e.g. because MR activation results in NO release. However, eplerenone did not affect the Ang II response in the presence of 1 μmol/L aldosterone. Thus, either these GPR30-counteracting effects occur in a MR-independent manner, or eplerenone is unable to block MR in vitro, for instance because the 3D structure of the MR, when located on the membrane, differs from its structure when located in the cytosol. Interestingly in this regard, a truncated variant of the MR, consisting of its C-terminal domains E and F only (MR EF), was still capable of inducing non-genomic effects, whereas the full-length MR was required for its genomic effects. In fact, MR EF sufficed for aldosterone-induced ERK1/2-activation via cSrc and EGFR, MR antagonists also bind to the MR section, but whether truncation alters their binding properties is currently unknown. Clearly, a truncated MR that responds to aldosterone but is no longer capable of binding MR antagonists could explain the lack of effect of MR antagonists.

Finally, GPR30 agonists at micromolar concentrations may desensitize the GPR30, thus no longer allowing potentiation. Although this mechanism could explain the lack of Ang II potentiation at micromolar aldosterone concentrations, it cannot explain why 17β-oestradiol at micromolar concentrations annihilated the effects of Ang II, nor why aldosterone at such concentrations induced NO release from endothelial cells.

In the present study, EGFR antagonism with AG1478 fully blocked the combined Ang II-aldosterone/17β-oestradiol effect, although AG1478 did not affect the response to Ang II alone. This indicates
that the contractile Ang II response in the presence of aldosterone or 17β-oestradiol entirely depends on EGFR transactivation, and differs from the response without aldosterone, for instance because aldosterone induces AT1 receptor dimerization. Since GRP30 activation is known to result in EGFR transactivation, our data now support the occurrence of such transactivation following AT1 receptor–GRP30 interaction induced by both aldosterone and 17β-oestradiol. In support of this concept, EGFR are known to occur in vascular smooth muscle cells as well as endothelial cells, and aldosterone has been reported to promote EGFR expression. The AG1478 concentration required to block the combined Ang II/steroid effect was 10 μmol/L. Although this concentration has been applied by many other investigators, 10-fold lower concentrations have also been used. In our hands such low concentrations were without effect. Nevertheless, a non-specific effect is highly unlikely, since 10 μmol/L AG1478 did not affect the response to Ang II alone. Neither the ERK1/2 pathway nor the p38 MAPK pathway contributed to the combined Ang II–aldosterone constrictor response (or the response of Ang II alone), thereby demonstrating that the second messenger pathways resulting in vasoconstriction differ from those inducing inotropy, proliferation, migration, or senescence.

A limitation of our study is that most experiments have been done in HCMAs from cerebrovascular patients in whom large blood pressure fluctuations are likely to have occurred in the pre-terminal phase. Such fluctuations may have affected the expression of AT1 receptors, MR, and/or GRP30 in the coronary vascular bed, thereby explaining some of the variation observed in this study. In addition, all experiments were performed in the presence of indomethacin, deleting a role for constrictor and/or dilator prostaglandins in our experimental set-up. Although previous studies have indicated that prostaglandins may counteract the effects of Ang II in the rat coronary vascular bed, indomethacin did not affect basal tone in HCMAs, nor did it affect the constrictor and relaxant effects of various (ant)agonists in these vessels in previous studies. Nevertheless, a role for prostaglandins in the Ang II–aldosterone interaction cannot be totally excluded. Finally, the pre-incubation period with steroids (30 min) may have been too long to fully exclude nuclear mechanisms, and too short to allow nuclear effects to be exerted in full. Unfortunately, given the scarcity of the material we were unable to test various incubation times. However, given the acute nature of the synergistic/antagonistic effects observed in combination with Ang II, it seems reasonable to assume that these effects are non-genomic.

### 4.1 Perspective

Aldosterone and other steroids potentiate Ang II at (low) nanomolar concentrations via GRP30, and this effect disappears at higher steroid concentrations, or is even reversed into a blockade of Ang II-induced constriction. The latter may represent a protective mechanism required to overcome the consequences of excessive steroid activation, e.g. during heart failure. Obviously, high, micromolar aldosterone (or oestradiol) concentrations do not occur in plasma, even under pathological conditions, but such concentrations may be present at tissue sites. A biphasic response to aldosterone has been described before in humans, supporting the possibility that both mechanisms can be simultaneously operative in vivo. The current study now sheds light on the underlying mechanisms as well as on its possible concentration dependency. The EGFR dependency of the aldosterone-induced Ang II potentiation is in full agreement with a recent study in unilaterally nephrectomized mice with deficient EGFR activity, where the aldosterone-induced potentiation of Ang II was absent. Since MR antagonists do not (or at most partially) block the GRP30, our data imply that aldosterone synthesis inhibitors may exert additional effects on top of MR antagonists.

### Conflict of interest

none declared.

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### References

Interaction between angiotensin II and aldosterone


38. Saxena PR, Daniels AHJ. Prorenin induces intracellular signaling in cardiomyocytes independently of angiotensin II. Hypertension 2006;48:564–571.


