Cardiac aldosterone: origin and effects

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Cardiaal aldosteron: oorsprong en effecten

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To my parents

For my son

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List of abbreviations

ACE	angiotensin converting enzyme
Ang	angiotensin
Ang II	angiotensin II
AT_1	angiotensin II type 1
AT_2	angiotensin II type 2
BSA	body surface area
CF	coronary flow
CRCs	concentration response curves
CYP11B2	cytochrome P450 11ß-hydroxylase 2
DAG	1,2 diacylglycerol
DMSO	dimethylsulfoxide
EDHF	endothelium-derived hyperpolarizing factor
EPHESUS	eplerenone heart failure and survival study
ERK	extracellular signal-regulated kinase
HCA	human coronary artery
HCM	hypertrophic cardiomyopathy
HERG	human Ether-a-Go-Go-Related gene
11ßHSD2	11ß-hydroxysteriod dehydrogenase 2
IP_3	1,4,5-trisphosphate
IVS	interventricular septal thickness
L-NAME	N^{ω}–nitro-L-arginine methyl ester HCl
LV	left ventricular
LVEDP	left ventricular end-diastolic pressure
LVM	left ventricular mass
LVMI	left ventricular mass index
LVP	left ventricular pressure
MI	myocardial infarction
MR	mineralocorticoid receptor
NO	nitric oxide
NOS	nitric oxide synthase
RAAS	renin-angiotensin-aldosterone system
RAS	renin-angiotensin system
RALES	randomized aldosterone evaluation study
PKC	protein kinase C
ROS	reactive oxygen species
StAR	steroidogenic acute regulatory

Chapter 1

Introduction and Aim

Traditionally, treatment of heart failure and hypertension has been performed mainly on the basis of the renin-angiotensin system rather than the renin-angiotensin-aldosterone system (RAAS), with the assumption that aldosterone will be suppressed once angiotensin (Ang) II formation is blocked. However, aldosterone formation does not stay suppressed during prolonged angiotensin-converting enzyme (ACE) inhibitor therapy. After 3 months of therapy, aldosterone levels start to rise again and they continue to do so due to "Ang II reactivation" or "aldosterone escape" [1,2].

Two clinical trials, the Randomized ALdosterone Evaluation Study (RALES) [3] and the Eplerenone HEart failure and SUrvival Study (EPHESUS) [4], have indicated that the aldosterone antagonists spironolactone and eplerenone reduce mortality in patients with heart failure and systolic left ventricular dysfunction post-myocardial infarction (MI) on top of ACE inhibition. These results draw attention to the importance of aldosterone in the pathophysiology of cardiovascular disease.

Circulating and local renin-angiotensin-aldosterone system

The RAAS has been viewed conventionally as a circulating system, involved in the regulation of salt, fluid homeostasis and blood pressure. Kidney-derived renin cleaves liver-derived angiotensinogen to form Ang I in circulating blood (Figure 1). ACE, located at the luminal side of the endothelium, subsequently converts Ang I to Ang II. Ang II exerts its effects via stimulation of Ang II type 1 (AT_1) and type 2 (AT_2) receptors. Besides acting as a vasoconstrictor via AT_1 receptors, Ang II also stimulates the formation of the sodium-retaining hormone aldosterone. Aldosterone mediates its cellular effects by binding to the mineralocorticoid receptor (MR), a member of the steroid/thyroid/retinoid/orphan receptor family of transcription factors.

This classic concept has been updated in the past 2 decades. It is now believed that some or all of the components of the RAAS are synthesized locally in tissues such as the heart, vessel wall, and adrenal [5]. For instance, in the heart, Ang II is synthesized locally by cardiac ACE following uptake of renin and angiotensinogen from the circulation [6,7]. This Ang II subsequently stimulates cardiac AT_1 and AT_2 receptors.

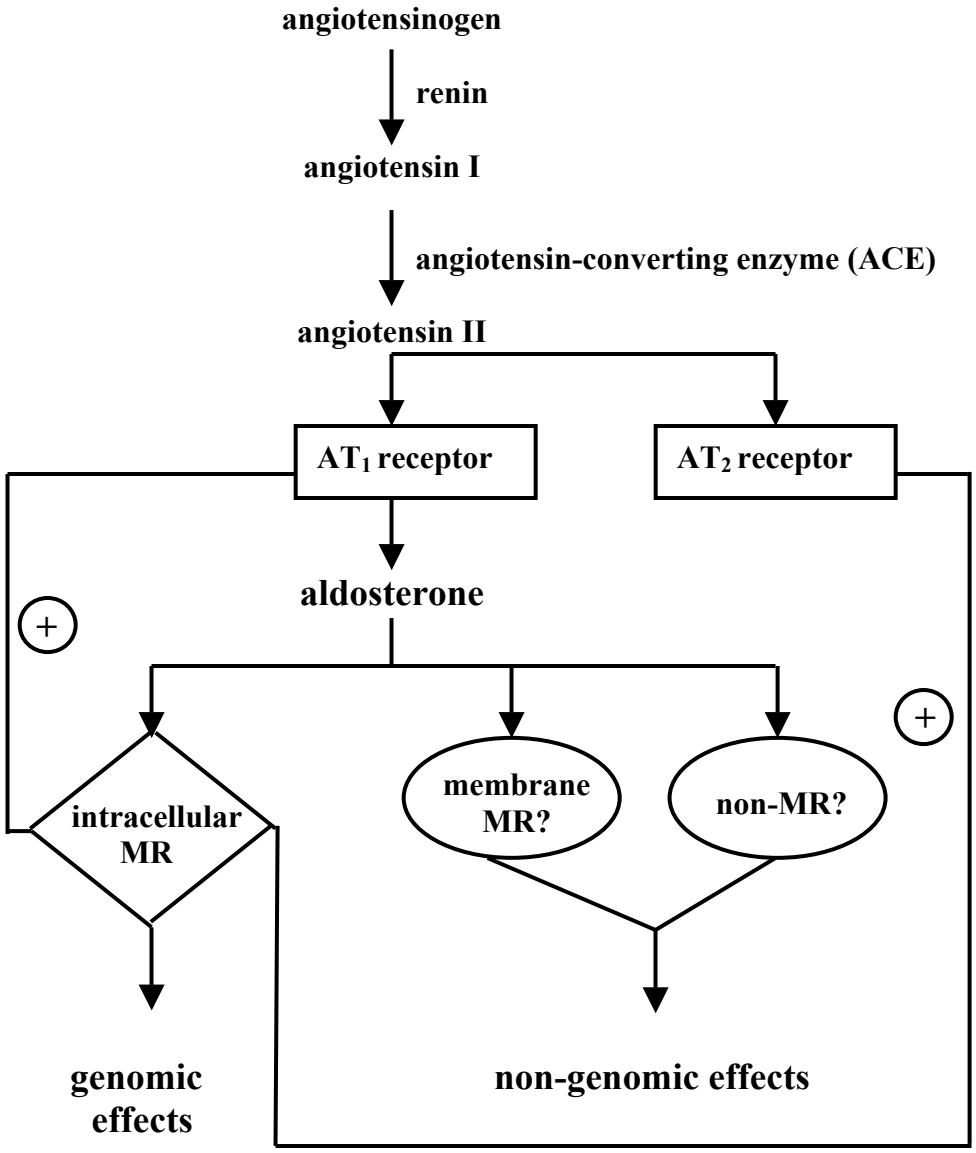

Figure 1 *The renin-angiotensin-aldosterone system. Angiotensin II activates angiotensin II type 1 and type 2 (AT_1 and AT_2) receptors. AT_1 receptor activation in the adrenal results in the synthesis and release of aldosterone, which subsequently exerts 'genomic' (after hours, involving protein synthesis) or 'non-genomic' (within minutes, not involving protein synthesis) effects through either mineralocorticoid receptors (MR) or other, as yet unidentified, receptors. MR may occur both intracellular and on the cell membrane. Aldosterone upregulates both AT_1 and AT_2 receptors, and AT_1 receptor activation also results in MR stimulation in an aldosterone-independent manner.*

Local synthesis of Ang II at cardiac tissue sites is in agreement with the observation that the beneficial effects of ACE inhibitors in heart failure are, at least in part, independent of their effect on blood pressure [8].

More recently, the MR has also been demonstrated in the heart, both at the mRNA and protein level [9]. Importantly, the enzymes required for the synthesis of aldosterone appear to be expressed in the human heart as well [10]. Together with the fact that Ang II is capable of increasing the aldosterone levels in isolated rat hearts and blood vessels [11,12], these data suggest that aldosterone, like Ang II, may be formed and act locally in the heart.

Aldosterone synthesis

Aldosterone, a steroid that was originally discovered in 1953, is secreted by the zona glomerulosa cells of the adrenal cortex. The kidney is the major target for adrenal aldosterone to increase sodium (and consequently water) reabsorption and potassium excretion. The production of aldosterone is regulated at two critical enzyme steps: (1) the formation of pregnenolone from cholesterol by the mitochondrial enzyme P450scc (side-chain cleavage) and (2) the conversion of corticosterone to aldosterone by cytochrome P450 11β-hydroxylase 2 (CYP11B2, 'aldosterone synthase'). Aldosterone synthesis in the adrenal cortex is regulated by Ang II, potassium and, more weakly, sodium and ACTH.

Extra-adrenal aldosterone synthesis has been proposed in the heart [12], brain [13] and vessel wall [11,14]. The rat heart expresses the steroidogenic acute regulatory (StAR) protein and aldosterone synthase, although at 100-fold lower levels than the adrenal [12,15]. The StAR protein facilitates intramitochondrial cholesterol transfer, the rate-limiting step of steroidogenesis. Aldosterone synthase expression has also been proposed in the human heart [10,16]. However, not all studies agree on the idea of local synthesis of aldosterone in the rat heart [17,18], and in humans, both extraction and release of aldosterone have been demonstrated across the coronary vascular bed [19,20].

Mineralocorticoid receptor: distribution and activation

MRs occur both in Na^+-transporting epithelia (e.g., kidney, colon) and non-epithelial tissues such as brain [21], heart (cardiomyocytes) [9], and blood vessels (endothelial and smooth muscle cells) [22-24]. The presence of MR in the cardiovascular system has been confirmed both at the mRNA and protein levels in animal models as well as in humans [24].

The classical MR-mediated effects of aldosterone are referred to as 'genomic' effects. These effects involve binding of aldosterone to intracellular MRs ($K_d \approx$ 1-2 nM), and the translocation of the steroid-MR complex to the nucleus, where it acts as a transcriptional regulator, inducing protein synthesis after several hours. Genomic effects can be inhibited by agents that block either transcription (e.g., actinomycin D) or translation (e.g., cycloheximide).

MRs bind mineralocorticoids and glucocorticoids with equal affinity. Yet, the circulating concentrations of glucocorticoids are several orders of magnitude higher than those of aldosterone. Selectivity of aldosterone binding to MR in the heart is achieved by co-expression of 11β-hydroxysteroid dehydrogenase 2 (11βHSD2). This enzyme converts cortisol and corticosterone into their non-MR-binding metabolites cortisone and 11-dehydrocorticosterone. In addition, the off-rate of aldosterone from the MR is five times lower than that of glucocorticoids [25].

Nongenomic effects of aldosterone

In addition to its genomic effects, which occur after hours, aldosterone also exerts rapid effects (within minutes) in various tissues, e.g. heart and vasculature. These effects are usually described as 'nongenomic' effects, since they do not involve DNA-directed, RNA-mediated protein synthesis. For instance, aldosterone rapidly affects cardiac inotropy, and facilitates both vasodilation and vasoconstriction [26-31]. In many (but not all) cases, these effects could not be blocked by MR antagonists, and therefore the existence of a novel (membrane-associated?) aldosterone receptor has been proposed [32,33]. However, despite numerous efforts in the past decade, no convincing data toward the characterization of a membrane receptor for aldosterone have been put forward

[33,34]. Consequently, it is not unlikely that these effects after all are also being mediated via the classical intracellular MR [33]. If so, an explanation must be provided for the lack of effect of MR antagonists toward the aldosterone-induced actions in vitro.

These actions involve activation of the phospholipase C – protein kinase C (PKC-inositol 1,4,5-trisphosphate (IP_3)-1,2 diacylglycerol (DAG) pathway, which leads to an increase in intracellular Ca^{2+} and stimulation of the Na^+/H^+ exchanger [26,30,33,35-37]. The latter causes a rise in intracellular Na^+, which subsequently activates Na^+/K^+-ATPase. Interestingly however, when the increase in intracellular Na^+ is prevented, aldosterone decreases Na^+/K^+-ATPase activity in a PKC-dependent manner [30]. Thus, aldosterone may exert both positive and negative inotropic effects. Other second messenger pathways that have been linked to the rapid effects of aldosterone include MAP kinases, reactive oxygen species (ROS) and the epidermal growth factor receptor [27,38].

Adverse effects of aldosterone in the cardiovascular system

Substantial evidence has emerged showing that aldosterone induces adverse effects in the cardiovascular system. The co-expression of 11βHSD2 and MR in human heart and blood vessels [9,38,39], albeit at low levels, supports the concept that these organs possess the cellular machinery required for direct aldosterone action, irrespective of the source of this aldosterone.

Endothelial dysfunction. Aldosterone increases the volume and stiffness of endothelial cells and induces gap formation, allowing irregular diffusion pathways for large particles [22,23]. This mechanism could contribute to endothelial dysfunction observed in hyperaldosteronism. The normalization of endothelial function by spironolactone in patients with heart failure supports this concept [40,41].

Oxidative stress, inflammation and fibrosis. A growing number of studies supports a specific role of the MR as a mediator of oxidative stress and subsequent inflammation and fibrosis. Elevations in circulating aldosterone are accompanied by a

pro-inflammatory/fibrogenic vascular phenotype [42-44], and since this phenomenon can be blocked by both spironolactone and anti-oxidants [42], it appears that aldosterone, via MR, induces oxidative stress. Indeed, aldosterone upregulates various subunits of NADPH oxidase and induces ROS generation in mononuclear and vascular smooth muscle cells [27,42,43,45]. In addition, aldosterone stimulates collagen synthesis in cardiac fibroblasts [46]. Consequently, aldosterone-induced cardiac fibrosis may be due to both direct effects in the heart (mediated via fibroblasts) and indirect peripheral effects (mediated via oxidative stress-activated mononuclear cells) [43].

Arrhythmias. MR blockade, in addition to standard therapy, reduced sudden death in RALES and EPHESUS [3,4]. The mechanism responsible for this favorable effect probably relies on both renal changes in electrolyte excretion and myocardial fibrosis inhibition. In addition, conditional MR overexpression in the mouse heart, in the absence of aldosteronemia, was found to result in severe ventricular arrhythmias [47]. Apparently therefore, cardiac MR trigger arrhythmias also directly, thus providing an additional mechanism through which MR antagonists reduce sudden death. In further support of this possibility, spironolactone improved electrophysiological parameters such as QT interval dispersion [48], and, in combination with the ACE inhibitor fosinopril, reduced the arrhythmic score post-myocardial infarction [49].

Interaction with angiotensin II. Ang II stimulates the synthesis and release of aldosterone in the adrenal, and thus it is not surprising that MR blockade and/or aldosterone synthase inhibition exert beneficial effects in Ang II-dependent models [17,50]. Remarkably however, aldosterone also appears to exert its effects, at least in part, via Ang II (or its receptors), and both agonists, when applied together, act synergistically [27,51,52] (Figure 1). Moreover, in human coronary artery smooth muscle cells Ang II stimulated MR-mediated gene expression in an aldosterone-independent manner, suggesting MR activation by post-translational modifications such as phosphorylation [38].

Aim of this thesis

One of the key insights into the pathphysiology of heart failure over the past twenty years has been the understanding that neurohormonal activation is an important and modified aspect of the disease process. Therefore, current treatment is not only directed toward the relief of symptoms but also toward inhibition of neurohormonal activation, in particular inhibition of the RAAS. The benefit of MR antagonists on top of ACE inhibition is not yet fully understood. Their effects cannot be attributed solely to blockade of the renal MR-mediated effects on blood pressure, and it is therefore now generally assumed that aldosterone also exerts actions in extrarenal tissues.

In the present study, we first set out to study the origin and site of production of Ang II in the adrenal (Chapters 2 and 3). Recent studies have identified a second renin transcript in adrenal tissue that gives rise to a truncated prorenin representing a cytosolic form of renin [53]. This truncated prorenin is transported to the mitochondria, where it may contribute to aldosterone biosynthesis through stimulation of intracellular Ang II production.

Next, we evaluated the effects of aldosterone on inotropy and flow in the rat Langendorff heart (Chapters 4 and 5). We distinguished genomic and nongenomic effects by using the MR antagonists spironolactone and eplerenone, taking into consideration that these antagonists may also exert effects of their own [29,54]. Furthermore, we studied the effects of aldosterone on DNA and collagen synthesis in cultured cardiac cells, and, in view of the pro-arrhythmogenic effects of aldosterone in the heart [47], we investigated the cardioprotective effect of spironolactone and eplerenone during ischaemia and reperfusion. In all rat heart studies, a comparison was made with the effects of Ang II, because of the possible synergy between the two agonists. We also quantified the kinetics of aldosterone in the isolated heart, in order to address the origin of cardiac aldosterone.

Finally, we studied the effects of aldosterone and its antagonists in human myocardial trabeculae and coronary arteries, both with and without Ang II, and we determined the second messenger pathways that mediated these effects (Chapter 6). We also compared the cardiac tissue levels of aldosterone and renin in healthy and diseased human hearts, and we investigated the association of the aldosterone synthase gene

(CYP11B2) C-344T polymorphism with cardiac hypertrophy in subjects with hypertrophic cardiomyopathy (Chapter 7).

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Chapter 2

Is angiotensin II made inside or outside of the cell?

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Is Angiotensin II Made Inside or Outside of the Cell?

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Angiotensin synthesis at tissue sites is well-established, and depends largely, if not completely, on kidney-derived renin. The exact tissue site of angiotensin generation (extracellular fluid, cell surface, intracellular compartment) is still being debated. In this review, we discuss the various possibilities, taking into consideration the intracellular occurrence/absence of prorenin, renin, angiotensinogen, angiotensin-converting enzyme, and angiotensin receptors; the local activation of prorenin to renin; the differences between in vivo and in vitro studies; and the methodologic difficulties related to angiotensin measurements. It is eventually concluded that angiotensin generation at tissue sites occurs extracellularly, most likely on the cell surface.

Introduction

It is now well-established that angiotensin (Ang) I and II are synthesized at tissue sites. In fact, most, if not all, tissue Ang II is synthesized locally from locally produced Ang I [1,2], and the beneficial effects of renin-angiotensin system (RAS) blockers are most likely due to interference with this Ang II rather than the Ang II in circulating blood [3]. Although originally it was thought that the renin required for such local synthesis was also locally produced, studies in nephrectomized animals provided convincing evidence that this is not the case [4–8]. Thus, in many tissues, in particular heart and vessel wall, local Ang I synthesis depends on kidney-derived renin. In addition, prorenin, the inactive precursor of renin, may contribute to Ang I generation at tissue sites [9,10]. This would, however, require its local activation following uptake from the circulation. Studies in transgenic animals displaying (inducible) prorenin expression in the liver support this concept [11,12]. The question, therefore, arises: Where does this circulating (pro)renin act: extra- or intracellularly? In case of the latter, the intracellular occurrence of both angiotensinogen and angiotensin-converting enzyme (ACE) are required to allow intracellular Ang II generation.

Intracellular Presence of Renin

After establishing the concept that tissue renin is kidney-derived, attention is focused on how tissues sequester renin and/or prorenin from the circulation. Diffusion into the interstitial space is one possibility [13,14], but evidence has also been obtained for the existence of (pro)renin receptors [15••,16–18]. Two such receptors have now been identified: the mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptor [9,16] and a recently cloned "renin receptor" [15••], which is identical to the vacuolar adenosine triphosphate (ATP) synthase membrane sector-associated protein M8-9 [19]. Both receptors bind renin and prorenin equally well. M6P/IGFII receptors internalize M6P-containing renin and prorenin, and prorenin internalization is followed by intracellular activation (through proteolytic cleavage of the prosegment) of prorenin to renin. Such activation, however, did not result in intracellular angiotensin generation in cardiac and vascular cells, mainly because these cells did not contain or sequester angiotensinogen [9,20••]. Therefore, this receptor may actually be a clearance receptor for (pro)renin.

Binding to the "renin receptor" facilitates Ang I generation on the cell surface, not only following binding of renin, but also following binding of prorenin. The latter suggests that prorenin, once bound to this receptor, undergoes a conformational change (uncovering the active site) or is proteolytically activated to renin.

Finally, a third, as yet unidentified, (pro)renin receptor has been proposed, based on observations that rat cardiomyocytes internalize prorenin (but not renin) in an M6P/IGFII-independent manner [18]. Interestingly, internalization of mouse *ren-2*^{d}, but not rat, prorenin via this unidentified mechanism resulted in angiotensin generation. The authors attributed this difference to the fact that mouse *ren-2*^{d} prorenin is unglycosylated, because glycosylation will determine the pathway of internalization and/or the degree of intracellular activation. However, care must be taken when interpreting these results, because the intracellular Ang I and II levels that were detected by direct radioimmunoassay after adding mouse *ren-2*^{d} prorenin were 100 to 200 pg/mg protein—*ie*, approximately three to four orders of magnitude higher than the in vivo levels of Ang I and II in the heart (5–20 pg/g tissue) [4–6]. Such high levels seem unlikely in view of the low angiotensinogen levels in the rat heart [8,21], and future studies should, therefore,

re-evaluate these high levels, preferably using high-performance liquid chromatography separation prior to the radioimmunoassay. Such studies should simultaneously address the intracellular occurrence of angiotensinogen and ACE.

In summary, in cells that do not synthesize (pro)renin, binding and internalization of both renin and prorenin results in the accumulation of these RAS components in intracellular compartments. The consequences of this uptake are not yet entirely clear, and one possibility is that it reflects clearance.

Cells that do synthesize (pro)renin (*eg*, renal juxtaglomerular cells and adrenal glomerulosa cells) secrete renin from storage granules, and release prorenin constitutively. In addition, renin-synthesizing cells in adrenal, brain, and heart (but not kidney) express an alternative renin transcript, called exon 1A renin [22,23]. This transcript codes for a truncated prorenin, which is not targeted to the secretory pathway, but remains intracellular. Its function is currently unknown. In the adrenal, it is transported to mitochondria [22]. Although its messenger RNA levels are elevated in the heart after myocardial infarction [23], the actual protein has not yet been demonstrated in cardiac tissue. The lack of renin-mediated Ang I–generating activity in cardiac tissue following a bilateral nephrectomy [4,8], as well as the fact that the Ang I–generating activity in hearts of nephrectomized animals does not increase following procedures that activate prorenin [4,8], suggest that truncated prorenin, if present in the heart, is not involved in angiotensin generation. The strong relationship between plasma and cardiac Ang I–generating activity under normal and pathologic conditions [21,24] also argues against the idea that locally synthesized truncated prorenin contributes to cardiac Ang I generation.

Intracellular Presence of Angiotensinogen, Angiotensin-converting Enzyme, and Angiotensin Receptors

Data on the intracellular presence of angiotensinogen and ACE are scarce. Angiotensinogen-synthesizing cells normally secrete angiotensinogen into the extracellular space, without storing it intracellularly. The tissue levels of angiotensinogen in non–angiotensinogen-synthesizing tissues are compatible with the idea that the presence of angiotensinogen is limited to the extracellular fluid—*ie*, blood and interstitial fluid [4,8,21,24]. Intracellular accumulation will only occur under (artificial) conditions where the N-terminal sequence required for secretion is deleted [25]. Evidence for angiotensinogen internalization could not be obtained [9,26], and, thus, the intracellular presence of angiotensinogen observed by some authors is most likely related to its intracellular synthesis and subsequent secretion [27,28].

Intracellular dialysis of Ang I into hamster cardiomyocytes resulted in Ang II–induced effects on cell coupling, as evidenced by the suppression of these effects following simultaneous intracellular application of enalaprilat [29]. Although this finding is in agreement with the concept that ACE is present intracellularly, Beldent *et al.* [30] were unable to demonstrate ACE activity in the cytosol. Possibly, therefore, Ang II was generated on the cell surface following the release of intracellularly applied Ang I to the extracellular space. Alternatively, as enalapril also increased cell coupling in the absence of Ang I, its effects may have been nonspecific (*ie*, unrelated to Ang II). The modest effects of losartan compared with enalapril in the same experimental setup (increases in junctional conductance of 16% and 72%, respectively) support this latter possibility. A second possibility is that losartan has blocked endogenous intracellular Ang II [31]. Even if this is true, it cannot be concluded that this Ang II originated from an extracellular source prior to the removal of the myocytes from the hamster [31]. The blocking effects of losartan, both at baseline and after the addition of Ang II [32], are in full agreement with the idea that Ang II type 1 (AT_1) receptors occur intracellularly [33•,34,35], particularly because the effects of losartan were observed only after intracellular application and not after extracellular application of the AT_1 receptor antagonist [32].

Intracellular Presence of Angiotensin I and Angiotensin II

Studies in which the blood plasma, interstitial, and tissue levels of Ang I, as well as its subcellular distribution, were determined revealed that tissue Ang I is restricted to the extracellular fluid compartment [36,37]. Thus, intracellular Ang I generation seems unlikely. In contrast, numerous reports support the intracellular presence of Ang II in multiple organs, including brain, heart, adrenal, and kidney [37–41]. In fact, most, if not all, tissue Ang II appears to be cell-associated [36,37], and most of this angiotensin is of local origin. Because its subcellular distribution is indistinguishable from that of systemically infused ^{125}I-labeled Ang II (which is exclusively of extracellular origin) [37], the most likely source of this cell-associated Ang II is the extracellular compartment. AT_1-receptor antagonists greatly reduce the tissue accumulation of infused Ang II, thereby suggesting that AT_1 receptor-dependent endocytosis underlies this uptake [36,37,39,40]. Extracellular Ang II is rapidly degraded by angiotensinases ($t_{1/2}$ < 1 minute) [36], whereas receptor-bound Ang II has a half life > 10 minutes [39]. Thus, under conditions in which complete AT_1-receptor blockade is achieved, tissue Ang II levels should decrease. The data of Mazzolai *et al.* [42••] fully support this concept. Furthermore, tissues with the highest AT_1 receptor density display the highest Ang II levels [39].

Taken together, therefore, the currently available in vivo data strongly suggest that generation of Ang I and II occurs extracellularly. The most likely site for tissue angiotensin generation, in view of the fact that both ACE and renin

receptors occur on the cell membrane, is the cell surface. Cell-surface generation is supported by studies revealing that membrane-bound renin cleaves angiotensinogen much more efficiently than soluble renin [15••] and that Ang II-mediated effects occur at 100-fold lower extracellular Ang II levels during prorenin + angiotensinogen application than during Ang II application [20••]. Apparently, angiotensin generation on the cell surface results in high Ang II levels in the micro-environment of AT receptors, allowing immediate binding of Ang II to its receptors rather than "loss" of Ang II into the extracellular space. Such efficient Ang II generation will not occur when non–membrane-bound enzymes, such as chymase, are involved [43], and this may explain why such enzymes do not contribute to Ang II synthesis in vivo.

In vitro data showing that cultured cells (mainly cardiomyocytes) release Ang II, particularly after stretch [44], appear to contradict the conclusion discussed earlier. However, the Ang II levels measured in these experiments were up to 1000-fold higher than the in vivo levels [45] and could not always be confirmed by others [26,46•]. An argument for stretch-induced Ang II release, even when such release could not be demonstrated, has always been that AT_1 receptor antagonists block the stretch-induced hypertrophic response. Recently, however, Zou *et al.* [46•] demonstrated that mechanical stress activates AT_1 receptors without the involvement of Ang II. Most likely, therefore, Ang II release from intracellular sources in cell culture studies is a methodologic artefact.

Is Angiotensin II An Intracrine Hormone?

The mere fact that Ang II, when applied [32,35] or expressed [33•,34] intracellularly, exerts effects is, of course, by no means evidence for its intracellular synthesis. Similarly, the intracellular presence of Ang II does not prove that Ang II is synthesized in the cell, because, as discussed earlier, it may simply reflect AT_1 receptor-mediated internalization. In fact, signaling is believed to depend, at least in part, on internalization [47], and thus, it is not surprising that Ang II, when applied or expressed intracellularly, exerts effects: it will stimulate internalized receptors. Internalization is also required to explain the recently reported interaction between Ang II and aldosterone [48,49], assuming that this involves intracellular mineralocorticoid receptors. Ré [50] defined an "intracrine" hormone not only as a hormone that is synthesized and acts intracellularly, but also as a hormone that is synthesized or secreted by a cell and then taken up by that same cell to act in the intracellular space. Therefore, even Ang II which, after its extracellular synthesis on the cell surface, is internalized and acts intracellularly, can be considered to be an intracrine hormone.

Conclusions

Although renin, Ang II, and AT_1 receptors can be demonstrated intracellularly, evidence for the intracellular presence of angiotensinogen, Ang I, and ACE is lacking. Rather, the tissue levels of both Ang I and angiotensinogen, when compared with their levels in blood and interstitial fluid, do not allow the conclusion that these components are cell-associated. Furthermore, the subcellular distribution of endogenous Ang II is indistinguishable from that of exogenously applied ^{125}I-Ang II. Consequently, Ang II synthesis must occur extracellularly, for instance on the cell surface, where both renin receptors and ACE are located. Subsequent internalization of Ang II via AT_1 receptors is not only a prerequisite for Ang II to induce a full response, but could also explain why both Ang II and AT_1 receptors can be found intracellularly. The latter probably also underlies ex vivo cell culture studies showing effects of intracellularly applied Ang II and/or AT_1 receptor antagonists. Finally, the intracellular presence of renin in cardiac and vascular cells most likely represents a clearance phenomenon, whereas the function of intracellular truncated (exon 1A) prorenin in adrenal mitochondria remains to be determined.

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Chapter 3

Adrenal angiotensin: origin and site of generation

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Adrenal Angiotensin: Origin and Site of Generation

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Background: Circulating angiotensin (Ang) II accumulates in adrenal tissue via binding to Ang II type 1 (AT_1) receptors, reaching levels that are 15 to 20 times higher than in blood. Adrenal tissue contains a second renin transcript that gives rise to a truncated prorenin representing a cytosolic form of renin. Here we investigated what percentage of adrenal Ang II originates at adrenal tissue sites, and whether intracellular renin contributes to adrenal angiotensin production.

Methods: Concentrations of endogenous and iodine-125 (^{125}I)–labeled Ang I and II were measured in adrenal tissue and blood from pigs after ^{125}I-Ang I infusion.

Results: In the adrenal tissue in all animals, ^{125}I-Ang I was undetectable. In untreated pigs, adrenal ^{125}I-Ang II was 17 $\pm$ 1 times arterial ^{125}I-Ang II, and tissue Ang I and II were 5 $\pm$ 1 and 388 $\pm$ 40 times higher than plasma Ang I and II. The AT_1 receptor antagonist eprosartan reduced adrenal ^{125}I-Ang II accumulation by 80%, and increased plasma Ang II to a greater degree than tissue Ang II. As a consequence, eprosartan equally reduced the tissue/plasma concentration ratios of both Ang II and ^{125}I-Ang II. Captopril did not alter ^{125}I-Ang II accumulation, and acutely, but not chronically, reduced the adrenal Ang II/I ratio.

Conclusions: More than 90% of adrenal Ang II originates at adrenal tissue sites. Local adrenal Ang II generation occurs extracellularly and is followed by internalization via AT_1 receptor-mediated endocytosis. Enhanced angiotensin generation, combined with incomplete AT_1 receptor blockade and the large adrenal AT_1 receptor reserve, explains why eprosartan increased rather than decreased adrenal Ang II. Our data do not support angiotensin generation by truncated prorenin. Am J Hypertens 2005;18: 1104–1110 © 2005 American Journal of Hypertension, Ltd.

Key Words: Adrenal, angiotensin, intracellular, receptor, renin.

Adrenal tissue contains high levels of angiotensin (Ang) II,[1] and it is generally believed that this Ang II originates from local production within adrenal tissue. All components required to generate Ang II locally are present in the adrenal[2–5] and in particular renin does not disappear from adrenal tissue after a bilateral nephrectomy.[3,5,6] This finding, which contrasts with the complete disappearance of renin from cardiac and vascular tissue after nephrectomy,[7,8] supports the concept of kidney-independent renin (and Ang II) synthesis in the adrenal gland. Moreover, adrenal renin levels, unlike plasma renin levels, correlate well with aldosterone production,[9,10] thereby confirming the concept that locally synthesized (in addition to circulating) Ang II affects adrenal aldosterone synthesis.

Although renin is considered to be a secretory protein (generating Ang I extracellularly), recent studies suggest that adrenal tissue contains a second renin transcript that gives rise to a truncated prorenin representing a cytosolic form of renin.[4] This truncated prorenin is transported into adrenal mitochondria, where it may contribute to aldosterone biosynthesis.[4] Other mechanisms resulting in the intracellular presence of renin include the internalization of renin or prorenin or both via either unidentified or mannose 6-phosphate/insulin-like growth factor II receptors,[11–13] and the subsequent intracellular activation of prorenin to renin.

Urata et al,[2] in a subcellular fractionation study, were unable to co-localize renin, angiotensinogen, and angiotensin-converting enzyme (ACE) in adrenal cells, and thus proposed that adrenal angiotensin production occurs by extracellular means. However, their study did not include the high-density fractions containing mitochondrial truncated prorenin. Subcellular fractionation also demon-

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strated that >95% of adrenal Ang II is cell associated.[14] Although this could suggest that intracellular Ang II synthesis does occur, it might also be explained on the basis on internalization of extracellularly generated Ang II. In support of the latter, circulating Ang II accumulates in adrenal tissue through Ang II type 1 (AT_1) receptor-mediated endocytosis, reaching steady-state levels in adrenal tissue that are 15 to 20 times higher than its levels in arterial plasma.[15] Accumulation of Ang II was also observed in kidney and heart, although at a much lower level.[15,16] Thus, the question arises what percentage of adrenal Ang II actually originates at adrenal tissue sites, and what percentage is taken up from blood.

It was the aim of the present study to determine the origin of adrenal Ang II. In addition, we investigated the possibility of intracellular Ang II production in the adrenal by truncated prorenin. All questions were addressed in a study in pigs by measuring the adrenal and plasma levels of iodine-125 (^{125}I)–labeled and endogenous Ang I and II during systemic ^{125}I-Ang I infusions. Because ^{125}I-labeled and endogenous angiotensins are pharmacokinetically indistinguishable,[17] a comparison of the levels of radiolabeled and endogenous angiotensins under various conditions will not only provide detailed information about the contribution of circulating Ang I and II to the adrenal angiotensin content but also, in view of the fact that adrenal ^{125}I-Ang II is by definition of extracellular origin, will shed light on the site of adrenal angiotensin generation.

Methods

Animals

All experiments were carried out under the regulations of the Animal Care Committee of the Erasmus Medical Center, Rotterdam, The Netherlands, in accordance with the "Guide for the Care and Use of Laboratory Animals" as published by the United States National Institutes of Health. A total of 45 Yorkshire × Landrace pigs (age 2 to 3 months, weight 25 to 30 kg) of either sex were included in the study. Five animals were untreated, and four pigs were treated with captopril (25 mg twice daily) for 3 days. In the 36 remaining animals a suture was placed around the left circumflex coronary artery to examine the effect of coronary ligation on the cardiac levels of Ang II in these animals.[18] The left circumflex coronary artery was permanently ligated in 24 animals, whereas the suture was removed in 12 animals (sham operation). Of the permanently ligated animals, six received captopril (25 mg orally, twice daily), and five animals received the AT_1 receptor antagonist eprosartan (400 mg orally, twice daily; a kind gift of Dr. P.K. Weck, SmithKline Beecham, Collegeville, PA). This dose of eprosartan blocks Ang II–induced pressor responses by >95%.[18] Treatment was started 12 to 24 h after ligation and was continued for 3 weeks. Coronary ligation did not alter the adrenal or plasma angiotensin levels (data not shown), and the data of all untreated pigs were therefore combined into one control group.

Instrumentation and ^{125}I-Ang I Infusion

The ^{125}I-Ang I infusions were given to all animals. In the untreated animals that received a suture around the left circumflex coronary artery, the infusions were given 1, 3, or 6 weeks after (sham) coronary ligation, and in the treated animals the infusions were given at the end of the treatment period, within 3 h after the last dose. Animals were prepared for administration of anesthetic and ^{125}I-Ang I, and for blood and tissue sampling as previously described.[15,18] After a stabilization period of 30 to 45 min after completion of instrumentation, animals were subjected to a 1-h infusion of ^{125}I-Ang I (~1 to 5 × 10^6 cpm/min) into the left ventricle. Both ^{125}I-Ang I and ^{125}I-Ang II reach steady-state levels in plasma within 10 min, and in adrenal tissue within 60 min, without affecting blood pressure.[15]

Collection of Blood and Tissue Samples

To measure the plasma levels of ^{125}I-labeled and endogenous Ang I and II, blood samples (5 to 10 mL) were taken from the aorta during the infusion of ^{125}I-Ang I as described before.[15] Plasma was stored at −70°C and assayed within 3 days.

Adrenal tissue was collected as follows. The heart was stopped by fibrillation while the ^{125}I-Ang I infusion was still running. The abdomen was opened by a longitudinal incision, and both adrenal glands were rapidly dissected. The adrenal glands were immediately frozen in liquid nitrogen and stored at −70°C.

Measurement of Angiotensins

The ^{125}I-labeled and endogenous Ang I and Ang II were measured as previously described,[7,15] using SepPak extraction and high-performance liquid chromatography (HPLC) separation. The concentrations of ^{125}I-labeled and endogenous angiotensins were not corrected for losses occurring during extraction and separation (approximately 20% to 30% in whole tissue homogenates and <10% in plasma[15]). For Ang I, the lower limit of detection was 0.5 fmol/mL of plasma and 1 fmol/g of tissue. For Ang II, the lower limit of detection was 0.3 fmol/mL of plasma and 0.5 fmol/g of tissue.

Calculations

In a previous study[15] we observed that the adrenal tissue/arterial plasma concentration ratio of ^{125}I-Ang II during ^{125}I-Ang I infusion was equal to that during ^{125}I-Ang II infusion. Thus the ^{125}I-Ang II that was present in adrenal tissue was derived from arterially delivered ^{125}I-Ang II and not, via conversion, from arterially delivered ^{125}I-Ang I. The adrenal tissue level of Ang II (fmol/g) that originates from angiotensin in the circulation was therefore calculated as follows:

[Ang II_{tissue}] originating from circulation

$$= R \times [\text{Ang } II_{art}], \quad (1)$$

in which [Ang II_{art}] is the steady-state concentration of Ang II in arterial plasma (fmol/mL), and R is defined by the equation:

$$R = [^{125}\text{I-Ang}II_{tissue}]/[^{125}\text{I-Ang}II_{art}], \quad (2)$$

in which [^{125}I-Ang II_{tissue}] and [^{125}I-Ang II_{plasma}] are the steady-state concentrations of ^{125}I-Ang II in tissue (cpm/g) and arterial plasma (cpm/mL), respectively.

Data Analysis

Data are expressed as mean ± SEM. Statistical analysis was by unpaired *t* test or one-way analysis of variance, followed by post hoc evaluation according to Tukey or Dunnet where appropriate. Statistical significance was accepted at $P < .05$.

Results

Untreated Pigs

Figures 1 and 2 show the plasma and tissue Ang I and II levels in untreated pigs ($n = 30$). Tissue Ang I was ~5 times higher than plasma Ang I, and tissue Ang II was ~400 times higher than plasma Ang II (Table 1). The ^{125}I-Ang I was not detectable in adrenal tissue (Fig. 3), whereas adrenal ^{125}I-Ang II was 17.4 ± 1.1 times higher than arterial plasma ^{125}I-Ang II (Table 1). From these data it can be calculated that all adrenal Ang I, and >90% of adrenal Ang II, originates from local production in the adrenal (Fig. 2). By far, ^{125}I-Ang II was the most important ^{125}I-labeled angiotensin metabolite in the adrenal (Fig. 3).

Effect of RAS Blockade

Acute captopril treatment ($n = 4$) lowered the Ang II levels in arterial plasma ($P < .05$) (Fig. 1). It tended to increase adrenal Ang I and to decrease adrenal Ang II (Fig. 2), but the differences were not significant. However, the Ang II/I ratio in adrenal tissue, a measure of ACE activity, did decrease by ~65% ($P < .05$) (Fig. 4). Acute captopril treatment also significantly reduced the Ang II/I and ^{125}I-Ang II/I ratios in plasma ($P < .01$ for both) (Fig. 4). During acute captopril treatment, adrenal ^{125}I-Ang I remained undetectable, and the tissue/blood plasma concentration ratio of ^{125}I-Ang II was identical to that in untreated pigs. The tissue/blood plasma concentration ratio of Ang II rose twofold ($P < .01$) (Table 1), and the contribution of circulating Ang II to the adrenal Ang II tissue levels decreased from 44 ± 10 to 15 ± 4 fmol/g ($P < .05$) (Fig. 2). Acute captopril treatment did not alter the amount of Ang II synthesized at adrenal tissue sites.

After chronic captopril treatment ($n = 6$), arterial plasma Ang I rose five- to tenfold ($P < .05$) (Fig. 1), and arterial plasma Ang II returned to control values. Adrenal angiotensin levels equaled those in untreated pigs (Fig. 2). The arterial plasma ^{125}I-Ang II/I and Ang II/I ratios remained reduced (Fig. 4), but the adrenal tissue Ang II/I ratio returned to control values. Chronic captopril treatment did not affect the adrenal uptake of arterial ^{125}I-Ang I and II (Table 1), and the contribution of circulating Ang II to adrenal Ang II (85 ± 30 fmol/g) was not different from that in untreated pigs. The amount of Ang I and II synthesized at adrenal tissue sites was also identical to that in untreated pigs (Fig. 2).

Chronic eprosartan treatment ($n = 5$) induced similar (fourfold) increases in plasma and adrenal Ang I ($P < .05$) (Figs. 1 and 2). It increased plasma Ang II to a larger degree than tissue Ang II (sixfold *v* threefold), and, as a consequence, the adrenal tissue/arterial plasma concentration ratio of Ang II was reduced by 70% ($P < .02$). Eprosartan did not significantly affect the ^{125}I-Ang II/I ratio in plasma (Fig. 4). It did reduce the adrenal uptake of

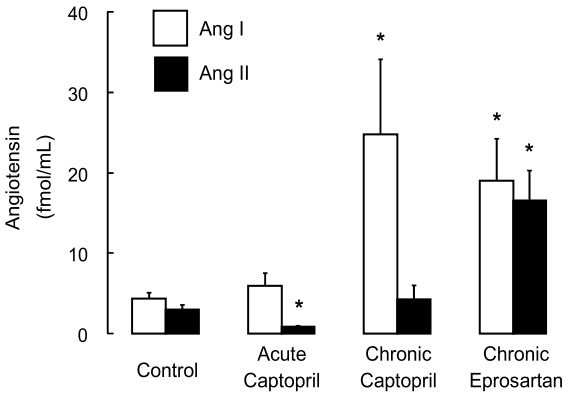

FIG. 1. Plasma levels of angiotensin (Ang) I and II in pigs that were untreated (control; $n = 30$), treated with captopril for 3 days (acute captopril; $n = 4$) or 3 weeks (chronic captopril; $n = 6$), or treated with eprosartan for 3 weeks (chronic eprosartan; $n = 5$). Data are mean ± SEM. *$P < .05$ *v* control.

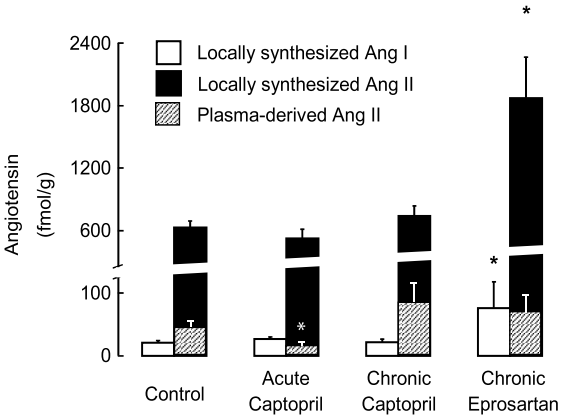

FIG. 2. Adrenal levels and origin of angiotensin (Ang) I and II in pigs that were untreated (control; $n = 30$), treated with captopril for 3 days (acute captopril; $n = 4$) or 3 weeks (chronic captopril; $n = 6$), or treated with eprosartan for 3 weeks (chronic eprosartan; $n = 5$). Adrenal Ang I did not originate in plasma. Data are mean ± SEM. *$P < .05$ *v* control.

Table 1. Adrenal tissue/arterial blood plasma concentration ratios of endogenous and radiolabeled angiotensin (Ang) I and II

Peptide	No Treatment (n = 30)	Acute Captopril Treatment (n = 4)	Chronic Captopril Treatment (n = 6)	Chronic Eprosartan Treatment (n = 5)
Ang I	5.1 ± 0.6	5.6 ± 1.5	1.5 ± 0.5	4.0 ± 2.4
Ang II	388 ± 40	757 ± 157*	397 ± 138	120 ± 20¶
^{125}I-Ang I	0	0	0	0
^{125}I-Ang II	17.4 ± 1.1	20.1 ± 4.5	20.7 ± 4.4	3.7 ± 0.8*

Data are mean ± SEM. Steady-state plasma ^{125}I-Ang I and II levels were 471 ± 22 and 302 ± 27 cpm/mL, 2906 ± 935 and 539 ± 289 cpm/mL, 620 ± 69 and 101 ± 17 cpm/mL, and 563 ± 82 and 385 ± 16 cpm/mL in the control, acute captopril, chronic captopril, and chronic eprosartan groups, respectively.

* $P < .01$; ¶ $P < .02$ *v* untreated.

^{125}I-Ang II by 79% ($P < .01$) (Table 1). The ^{125}I-Ang I remained undetectable in adrenal tissue in eprosartan-treated pigs. Eprosartan did not alter the contribution of circulating Ang II to the adrenal Ang II tissue levels (70 ± 25 fmol/g), and it greatly increased the synthesis of Ang I and II at adrenal tissues sites ($P < .05$) (Fig. 2).

Discussion

The present study shows that accumulation of circulating Ang II in the adrenal gland accounts for approximately 15 to 85 fmol Ang II/g wet weight. Such levels are comparable to or higher than the total amount of Ang II (per gram wet weight) in heart and kidney.[1,19,20] Yet, the total adrenal Ang II levels are >10 times higher, both with and without RAS blockade; thus, despite the considerable contribution of circulating angiotensins (supporting a role for circulating Ang II in the adrenal), the majority (>90%) of adrenal Ang I and II is derived from local origin, ie, it is synthesized at adrenal tissue sites, independently of either circulating Ang I or II.

Where does angiotensin generation at adrenal tissue sites occur? Intracellular Ang II synthesis is favored by previous studies showing that adrenal tissue contains a cytosolic form of renin (truncated prorenin)[4] and that >95% of adrenal Ang II is cell associated.[14] The current observation that adrenal Ang II levels increase during AT_1 receptor blockade also appears to support this concept. However, to put these findings into perspective, and as indicated in Fig. 5, the following points should be taken into consideration: 1) ^{125}I-Ang II accumulates at tissue sites through AT_1 receptor-mediated endocytosis,[15] 2) >95% of tissue ^{125}I-Ang II is cell-associated (ie, is either membrane-bound or intracellular)[14]; and 3) treatment with eprosartan increases circulating (extracellular) Ang II but not ^{125}I-Ang II. It is thus not surprising that eprosartan greatly reduces the ^{125}I-Ang II tissue/plasma concentration ratio. If adrenal Ang II, like ^{125}I-Ang II, is of extracellular origin, eprosartan should reduce the Ang II tissue/plasma concentration ratio to the same degree as that of ^{125}I-Ang II. In contrast, if adrenal Ang II is generated intracellularly, the Ang II tissue/plasma concentration ratio change after eprosartan should not parallel that of ^{125}I-Ang II. It may not change at all (Fig. 5), or it might even increase. Our data, showing an identical eprosartan-induced reduction in the tissue/plasma concentration ratio of Ang II and ^{125}I-Ang II (by 70% and 79%, respectively), strongly favor the first possibility. Thus Ang II synthesis at adrenal tissue sites occurs extracellularly, either in the interstitial fluid or on the cell surface.

Extracellular Ang II generation implies that tissue Ang I is restricted to the extracellular fluid compartment, ie, the interstitial fluid. The latter accounts for ~10% of adrenal tissue weight, and thus it can be estimated that the adrenal interstitial Ang I levels in untreated animals are ~200 fmol/mL, or 50 times its levels in circulating blood. The adrenal interstitial Ang II levels would be ~300 fmol/mL, provided that all of the 5% of adrenal Ang II that was previously found in the cytosolic/extracellular fluid fraction (after subcellular fractionation[14]) is present in interstitial fluid. Interestingly, these interstitial levels resemble the Ang I and II levels that were recently detected in the medium of cultured human adrenocortical NCI-H295 cells.[21]

Acute ACE inhibition with captopril lowered the adrenal Ang II/I ratio by approximately 65%. This reduction is less complete than the 80% to 90% decrease in the ratio of circulating Ang II and I, and it most likely reflects the inability of captopril, at the dose applied in this study, to fully block adrenal ACE. It does confirm, however, that orally applied captopril reaches adrenal tissue sites. Remarkably, during prolonged captopril treatment, the ratio of circulating Ang II and I remained reduced, whereas the adrenal Ang II/I ratio returned to normal levels. Similar observations were made previously in heart and kidney[18–20]; and collectively these data suggest that, chronically, ACE inhibition is overcome at tissue sites, either because of ACE upregulation in tissues or because other converting enzymes come into play.[1,22,23]

Chronic AT_1 receptor blockade increased both the circulating and adrenal angiotensin levels. In accordance with a previous study,[24] the increase in circulating Ang II was two to three times as large as the increase in adrenal

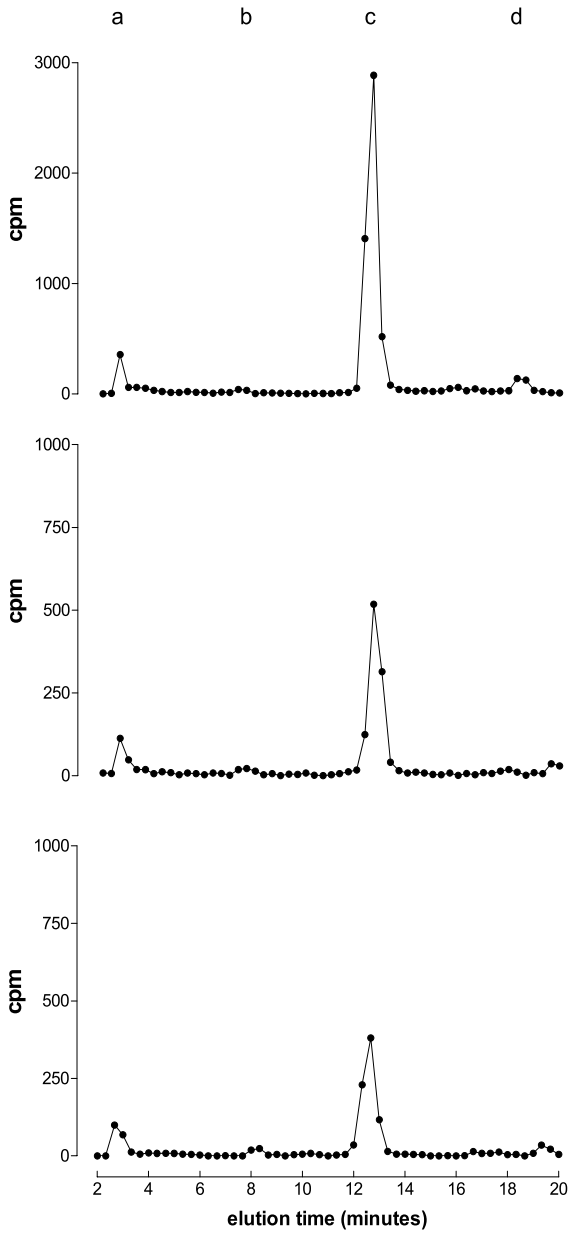

FIG. 3. High-performance liquid chromatography elution profile of iodine-125 (^{125}I)–labeled angiotensins (Ang) in adrenal tissue measured by gamma counting. Samples were obtained from a control pig (**top panel**), a captopril-treated pig (**middle panel**), and an eprosartan-treated pig (**bottom panel**), after a 1-h infusion of ^{125}I-Ang I. Retention times of radiolabeled standards: (**a**) ^{125}I-tyrosine; (**b**) ^{125}I-Ang–(4–8); (**c**) ^{125}I-Ang II; (**d**) ^{125}I-Ang I.

Ang II. The eprosartan-induced increases in plasma and adrenal Ang I were similar. Thus, for a given eprosartan-induced rise of Ang I, the rise in Ang II at adrenal tissue sites was smaller than in the circulation. Campbell et al have demonstrated that this is not due to a decrease in adrenal ACE.[24] Taking into account that AT_1 receptor binding protects Ang II against rapid metabolism,[15,25] a more likely explanation of this observation is that AT_1 receptor blockade has prevented locally generated Ang II from binding to AT_1 receptors, thereby decreasing its half life.

Adrenal AT_1 receptor density ranges from 20 to 100 pmol/g.[26,27] Thus, at the adrenal Ang II levels measured in untreated pigs in the present study (~0.5 pmol/g), >97.5% of adrenal AT_1 receptors is free. Eprosartan blocked approximately 80% of the adrenal AT_1 receptors (evidenced by the 79% reduction in adrenal ^{125}I-Ang II uptake). The remaining 20% would easily allow the threefold increase in the adrenal Ang II levels observed after eprosartan, and only under conditions where complete or near complete (>97.5%) AT_1 receptor-blockade is obtained a decrease in tissue Ang II levels will occur.[28]

How do AT_1 receptor blockers exert effects, if they increase rather than decrease the amount of AT_1 receptor–internalized Ang II per gram of tissue? To induce an effect, Ang II needs to stimulate a threshold number of AT_1 receptors per cell. Local Ang II generation (eg, on the cell surface) typically allows such a high degree of regional AT_1 receptor stimulation. During eprosartan treatment, this is no longer possible, although (due to the increased extracellular Ang II generation and the incomplete AT_1 receptor blockade), Ang II may still bind to AT_1 receptors without, however, reaching the threshold stimulation level in an individual cell. In other words, a high degree of regional AT_1 receptor stimulation is replaced by a low degree of overall AT_1 receptor stimulation. Although this

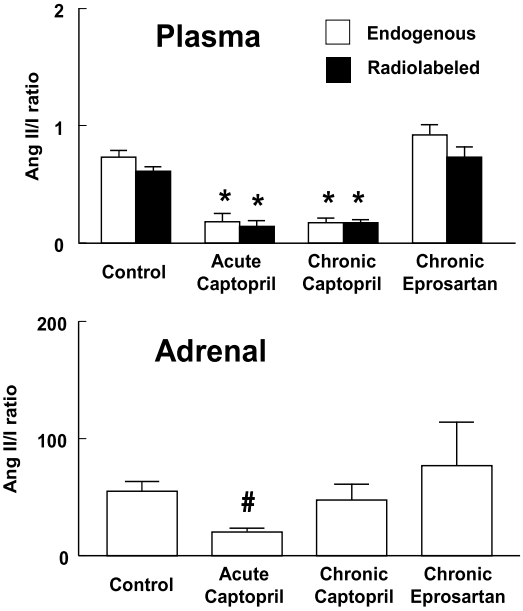

FIG. 4. Ratio of endogenous and radiolabeled angiotensin (Ang) II and I in plasma (**top panel**) and adrenal tissue (**bottom panel**) in pigs that were either untreated (control; n = 30), treated with captopril for 3 days (acute captopril; n = 4) or 3 weeks (chronic captopril; n = 6), or treated with eprosartan for 3 weeks (chronic eprosartan; n = 5). The ^{125}I-Ang I was undetectable in adrenal tissue, so the tissue ^{125}I-Ang II/I ratio could not be calculated. Data are mean ± SEM. *P < .01, #P < .05 v control.

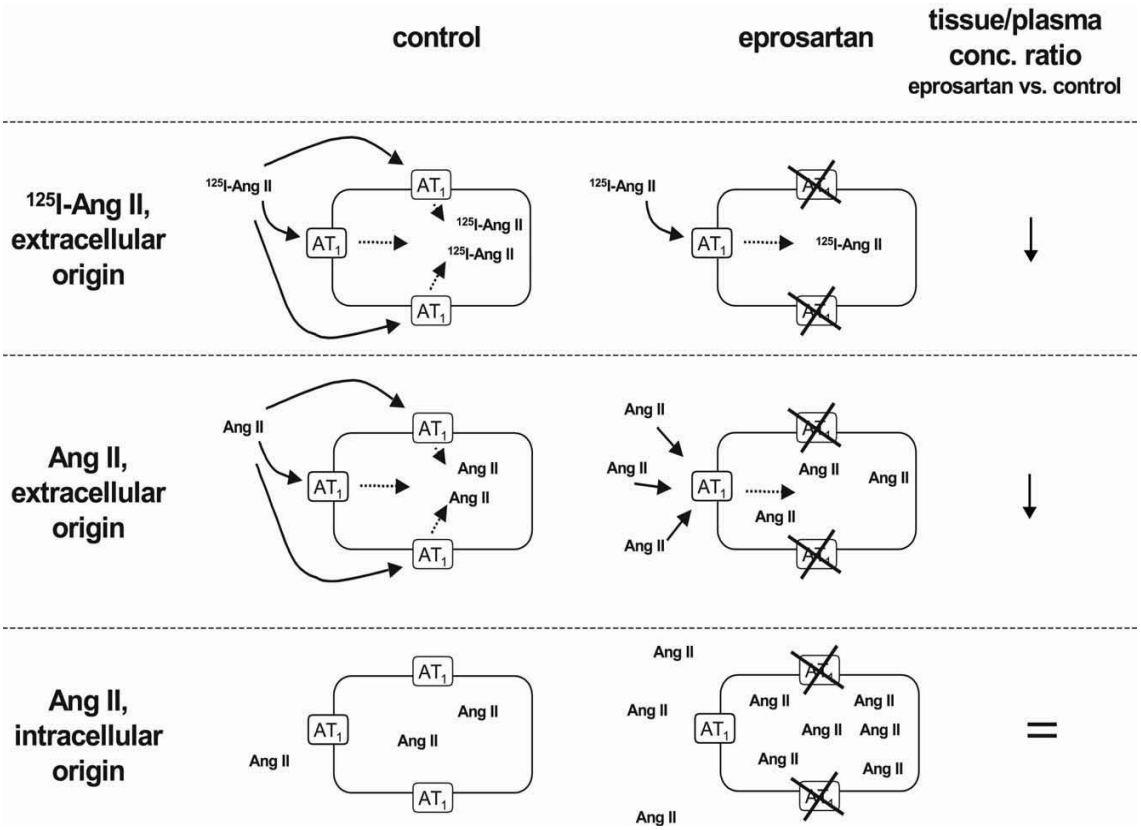

FIG. 5. Model distinguishing the consequences of eprosartan treatment on adrenal angiotensin (Ang) II content assuming either extra- or intracellular adrenal Ang II generation. For comparison, the well-known consequences of eprosartan treatment on tissue ^{125}I-Ang II (which is exclusively of extracellular origin) are also shown (for explanation, see Discussion). AT_1 = Ang II type 1 receptor; conc. = concentration.

will reduce the AT_1 receptor–mediated effects of Ang II, it does not necessarily lower the tissue levels of Ang II.

A similar mechanism may underlie the beneficial effects of ACE inhibition, which also appear to occur in the absence of a change in tissue Ang II content.[18,19] Normally, ACE generates Ang II in a highly efficient manner, in close proximity of AT_1 receptors.[22,29,30] Consequently, little Ang II needs to be generated to obtain maximal (regional) AT_1 receptor stimulation. During chronic ACE inhibition, the increase in Ang I generation will still allow Ang II generation, either by noninhibited ACE or by non–ACE such as chymase.[22,31] However, this type of Ang II generation is less efficient, because it does not result in a high level of regional AT_1 receptor stimulation. In particular, Ang II generated by chymase (which is localized in the adventitia[32]) will be subject to rapid metabolism in the interstitial space on its way to AT_1 receptors[25,33] and thus is less likely to result in a high regional AT_1 receptor occupancy. Again, a low overall AT_1 receptor occupancy will occur, below the minimum per cell required to induce an effect.

In conclusion, adrenal Ang II originates both in the circulation and at adrenal tissue sites. Local adrenal Ang II generation occurs extracellularly and is followed by internalization via AT_1 receptor–mediated endocytosis. The large adrenal AT_1 receptor density allows adrenal Ang II levels to increase further during eprosartan treatment, even when near-complete AT_1 receptor blockade is obtained. The absence of intracellular Ang II generation at adrenal tissue sites suggests that intracellular mitochondrial renin[3] may exert other, as yet unidentified, effects.

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Chapter 4

Genomic and nongenomic effects of aldosterone in the rat heart: why is spironolactone cardioprotective?

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Genomic and nongenomic effects of aldosterone in the rat heart: why is spironolactone cardioprotective?

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1 Mineralocorticoid receptor (MR) antagonism with spironolactone reduces mortality in heart failure on top of ACE inhibition. To investigate the underlying mechanism, we compared the actions of both aldosterone and spironolactone to those of angiotensin (Ang) II in the rat heart.

2 Hearts of male Wistar rats were perfused according to Langendorff. Ang II and aldosterone increased left ventricular pressure (LVP) by maximally 11 ± 4 and $9\pm2\%$, and decreased coronary flow (CF) by maximally 36 ± 7 and $20\pm4\%$, respectively. Spironolactone did not significantly affect LVP or CF.

3 In hearts that were exposed to a 45-min coronary artery occlusion and 3 h of reperfusion, a 15-min exposure to spironolactone prior to occlusion reduced infarct size (% of risk area) from 68 ± 2 to $45\pm3\%$, similar to the reduction ($34\pm2\%$) observed following 'preconditioning' (15 min occlusion followed by 10 min reperfusion) prior to the 45-min occlusion. Aldosterone exposure did not affect infarct size ($71\pm5\%$).

4 In cardiomyocytes, aldosterone decreased [^{3}H]thymidine incorporation maximally by $73\pm3\%$, whereas in cardiac fibroblasts it decreased [^{3}H]proline incorporation by $33\pm7\%$. Spironolactone inhibited both effects. Ang II increased DNA and collagen synthesis, and these effects were reversed by aldosterone.

5 In conclusion, aldosterone induces positive inotropic and vasoconstrictor effects in a nongenomic manner, and these effects are comparable to those of Ang II. Aldosterone reduces DNA and collagen synthesis *via* MR activation, and counteracts the Ang II-induced increases in these parameters. MR blockade reduces infarct size and increases LVP recovery following coronary artery occlusion. The MR-related phenomena may underlie, at least in part, the beneficial actions of spironolactone in heart failure.

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Keywords: Aldosterone; angiotensin; cardiomyocyte; collagen; DNA synthesis; ischaemia; Langendorff heart; mineralocorticoid receptor; spironolactone

Abbreviations: Ang, angiotensin; AT_1 receptor, angiotensin II type 1 receptor; CF, coronary flow; LVEDP, left ventricular end-diastolic pressure; LVP, left ventricular pressure

Introduction

Plasma aldosterone levels are elevated in patients with congestive heart failure, even when long-term ACE inhibitor therapy results in complete inhibition of membrane-bound ACE (Jorde *et al*., 2002). This elevation is not due to angiotensin (Ang) II generation by non-ACE enzymes such as chymase (MaassenVanDenBrink *et al*., 1999; Jorde *et al*., 2002; Tom *et al*., 2003), and thus factors other than Ang II (e.g., potassium, corticotropin and catecholamines) are responsible for the increased aldosterone production in heart failure during ACE inhibition (Weber, 2001).

Aldosterone excess has deleterious effects on cardiac function (Delcayre & Silvestre, 1999; Beggah *et al*., 2002; Sun *et al*., 2002; Ahokas *et al*., 2005), and treatment of patients experiencing severe heart failure with the aldosterone receptor antagonist spironolactone improves the morbidity and mortality on top of ACE inhibition (Pitt *et al*., 1999). The underlying mechanism of this beneficial effect is not entirely clear. Since the aldosterone receptor (mineralocorticoid receptor (MR)) occurs both in the kidney and extrarenal tissues like the heart and the vessel wall (Lombès *et al*., 1995; Mazak *et al*., 2004; Oberleithner *et al*., 2004), it is believed that spironolactone may exert its effects, at least in part, independently of the kidney. It has even been suggested that aldosterone, like Ang II (Danser *et al*., 1994; van Kats *et al*., 1998), is synthesized locally in the heart (Silvestre *et al*., 1998; 1999).

The MR-mediated effects of aldosterone are referred to as genomic effects. These effects involve binding of aldosterone to the intracellular MR ($K_d \approx 1$–2 nM) and the translocation of the steroid–MR complex to the nucleus, where it acts as a transcriptional regulator, inducing effects after several hours. In addition, rapid 'nongenomic' effects of aldosterone

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(occurring within minutes) have been described (Lösel *et al*., 2002). These effects are likely to be transmitted *via* specific membrane receptors. They occur at subnanomolar levels of aldosterone, and involve, among others, inositol 1,4,5-triphosphate (IP_3), protein kinase C and Ca^{2+} (Christ *et al*., 1993; 1995; Wehling *et al*., 1995). The identity of the receptor responsible for the nongenomic effects is currently not known.

The effects of aldosterone in the heart include inflammation, fibrosis, positive inotropy and coronary vasodilation (Campbell *et al*., 1993; Sun *et al*., 1993; Moreau *et al*., 1996; Barbato *et al*., 2002; Sun *et al*., 2002). Not all studies confirm these findings, however, nor has consensus been obtained on the receptor(s) mediating these effects. This is due to the fact that in some cases the results were obtained using only one concentration of aldosterone with or without spironolactone (Brilla *et al*., 1994; Moreau *et al*., 1996; Barbato *et al*., 2002), whereas in others the study involved *in vivo* treatment with spironolactone, which does not allow conclusions on the local cardiac effects of this drug (Rochetaing *et al*., 2003).

In the present study, we set out to distinguish the genomic and nongenomic effects of aldosterone in the heart. Using the isolated perfused rat Langendorff heart preparation, we evaluated the effects of aldosterone on left ventricular pressure (LVP) and coronary flow (CF), as well as its effects on infarct size and recovery of LVP and CF following ischaemia and reperfusion. In cultured neonatal rat cardiomyocytes and fibroblasts, we determined the effects of aldosterone on DNA and collagen synthesis. These effects were compared to the effects of aldosterone on DNA synthesis in aortic vascular smooth muscle cells (VSMCs). Genomic and nongenomic effects were distinguished using the MR receptor antagonist spironolactone. We also investigated the effects of Ang II, alone and in combination with aldosterone, because recent studies suggest that aldosterone exerts its effects, at least in part, *via* Ang II and/or Ang II type 1 (AT_1) receptors (Mazak *et al*., 2004; Michel *et al*., 2004; Xiao *et al*., 2004).

Methods

Drugs

Ang II, aldosterone, spironolactone and trypan blue were purchased from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands. Stock solutions of aldosterone (10 mM) and spironolactone (1 mM) were prepared in ethanol. All other chemicals were dissolved in distilled water.

Ethical approval

All experiments were performed under the regulations of the Animal Care Committee of the Erasmus MC, in accordance with the 'Guiding Principles in the Care and Use of Laboratory Animals' as approved by the American Physiological Society.

Experiments in Langendorff hearts

Male Wistar rats ($n = 66$, weight 300–340 g), obtained from Harlan, Zeist, The Netherlands, were anaesthetised with sodium pentobarbital (60 mg kg^{-1}, i.p.). Hearts were rapidly excised and cooled in ice-cold Krebs–Henseleit solution (composition in mM: NaCl 125, KCl 4.7, $NaHCO_3$ 20, NaH_2PO_4 0.43, $MgCl_2$ 1.0, $CaCl_2$ 1.3 and D-glucose 9.1; pH 7.4) until contractions stopped, and prepared for Langendorff perfusion. Continuously carbogen-gassed (95% O_2/5% CO_2) Krebs–Henseleit solution at 37°C was perfused immediately after cannulation of the aorta, at a constant perfusion pressure of 80 mmHg. A water-filled latex balloon was placed in the left ventricle *via* the left atrium to measure LVP. The volume of the balloon was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) of 5 mmHg during initial equilibration, and this volume was maintained throughout the experiment. Hearts were paced at 350 beats min^{-1}. CF was measured by an inline flow probe (Transonic Systems, Ithaca, NY, U.S.A.).

After a stabilization period of 15 min, 100 μl bolus injections were applied to construct dose–response curves to Ang II, vehicle (ethanol), aldosterone and/or spironolactone. In a second series of experiments, we evaluated the effects of aldosterone and spironolactone during ischaemia and reperfusion. Hearts were subjected to 45 min left anterior descending coronary artery occlusion, followed by 3 h of reperfusion. Occlusion was preceded by either no treatment (control), preconditioning (15 min of occlusion followed by 10 min of reperfusion), or a 15-min exposure to vehicle (0.01% ethanol, final concentration in perfusion buffer), aldosterone (100 nM) or spironolactone (100 nM). Vehicle, aldosterone and spironolactone remained present in the perfusion buffer throughout the remainder of the experiment. After the 3-h reperfusion period, area at risk and infarct size were determined as described before (Schoemaker & van Heijningen, 2000).

Experiments in cells

Primary cultures of neonatal Wistar rat (Harlan) ventricular cardiomyocytes and fibroblasts were prepared as described before (van Kesteren *et al*., 1997; Saris *et al*., 2002). Briefly, ventricles from newborn 1–3-day-old Wister rats were minced and cells of ventricles were isolated by eight subsequent trypsinization steps. Nonmyocytes were separated from myocytes by differential preplating. Myocytes were seeded in 24-well plates at $1.5–1.75 \times 10^5$ cells cm^{-2}, giving a confluent monolayer of spontaneously beating cells after 24 h. The preplated cells (fibroblast fraction) were passaged after 4 days to 24-well plates at 0.75×10^5 cells cm^{-2}. Myocytes and fibroblasts were maintained at 37°C and 5% CO_2–95% air in DMEM and Medium 199 (4 : 1), supplemented with 5% foetal calf serum (FCS), 5% horse serum, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}.

VSMCs were isolated from Sprague–Dawley rat thoracic aortas by enzymatic digestion as described previously (Haller *et al*., 1994). Cells were grown in SmGM-2 (Clonetics, Verviers, Belgium) supplemented with 5% FCS, and were passaged by harvesting with 0.05% trypsin/0.53 mM EDTA and seeding into 75 cm^2 flasks. For experiments, cells between passages 3 and 8 were seeded in 24-well plates and grown to confluency at 37°C and 5% CO_2–95% air.

Experiments were performed after the cells had been serum-deprived for at least 1 day. Before the start of each experiment, cells were rinsed three times with 1 ml warm (37°C) serum-free medium. Next, the cells were incubated for 24 h at 37°C with 400 μl serum-free medium (supplemented with 1% bovine serum albumin (BSA) in the case of myocytes and fibroblasts),

and containing Ang II, vehicle (0.1% ethanol), aldosterone and/or spironolactone. DNA and collagen synthesis rates were determined in duplicate by quantifying [^{3}H]thymidine and [^{3}H]proline incorporation, respectively, during the last 6 h of the above 24-h incubation period. Total cellular protein and DNA were quantified after solubilization as described before (van Kesteren *et al*., 1997), using BSA and salmon sperm as standard, respectively.

Data analysis

Data are expressed as mean ± s.e.m. and are shown either as percent change from baseline (LVP, CF) or relative to control values (DNA and collagen synthesis rates). Dose–response curves were analysed as described before (Tom *et al*., 2002) to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. Statistical analysis was carried out by ANOVA, followed by *post hoc* evaluation according to Tukey. *P*-values <0.05 were considered significant.

Results

Experiments in Langendorff hearts

Baseline values of LVP and CF were 80 ± 1.4 mmHg ($n=60$) and 12 ± 0.3 ml min^{-1}, respectively. Ang II ($n=6$) and aldosterone ($n=6$) dose-dependently increased LVP (pEC_{50} 7.4 ± 0.3 and 9.8 ± 0.4, respectively) and reduced CF (pEC_{50} 8.0 ± 0.7 and 8.7 ± 0.6, respectively) (Figure 1). Maximum effects of Ang II occurred within 2–3 min and lasted 5–6 min, whereas the maximum effects of aldosterone occurred within 1–2 min and lasted <2 min. Spironolactone ($n=6$) did not significantly affect LVP or CF (Figure 1). Vehicle ($n=6$) also did not affect CF, and decreased LVP at the highest two concentrations that were evaluated (corresponding to 0.1 and 1% ethanol in the bolus injection fluid, respectively). Spironolactone (10 μM, $n=6$) reduced the effect of aldosterone on LVP, without changing its pEC_{50}. Spironolactone did not alter the effects of aldosterone on CF.

In six (three control hearts, two aldosterone-pretreated hearts and one vehicle-pretreated heart) of the 36 hearts that were exposed to ischaemia plus reperfusion, we could not determine LVP and CF during reperfusion because of severe ventricular arrhythmias. These hearts were therefore excluded from the analysis. In the remaining 30 hearts, occlusion of the left anterior descending coronary artery reduced LVP to ≈30 mmHg and decreased CF by approximately 50% (Table 1). In control hearts ($n=6$), LVP and CF increased rapidy during the reperfusion phase (Table 1), stabilizing after approximately 20 min at 44 ± 3 and 86 ± 13% of pre-ischaemia values (Figure 2). LVEDP increased to 23 ± 5 mmHg. Pretreatment with aldosterone ($n=6$) or vehicle ($n=6$) did not affect LVP and CF recovery, nor did it prevent the rise in LVEDP. Preconditioning ($n=6$) as well as pretreatment with spironolactone ($n=6$) significantly enhanced recovery of LVP ($P<0.05$), but not of CF (Table 1 and Figure 2). Both procedures tended to reduce the rise in LVEDP ($P>0.05$). Ventricular fibrillation occurred within the first 10 min of reperfusion of all control hearts (Table 1). The incidence of fibrillation was reduced in hearts exposed to preconditioning or spironolactone, but not in hearts pretreated with aldosterone or vehicle. The area at risk was identical in all hearts (Table 1). Infarct size (expressed as a percentage of the area at risk) was 68 ± 2% in control hearts (Figure 2). Preconditioning and spironolactone similarly reduced infarct size ($P<0.05$), whereas infarct sizes in aldosterone- and vehicle-treated hearts were not different from control.

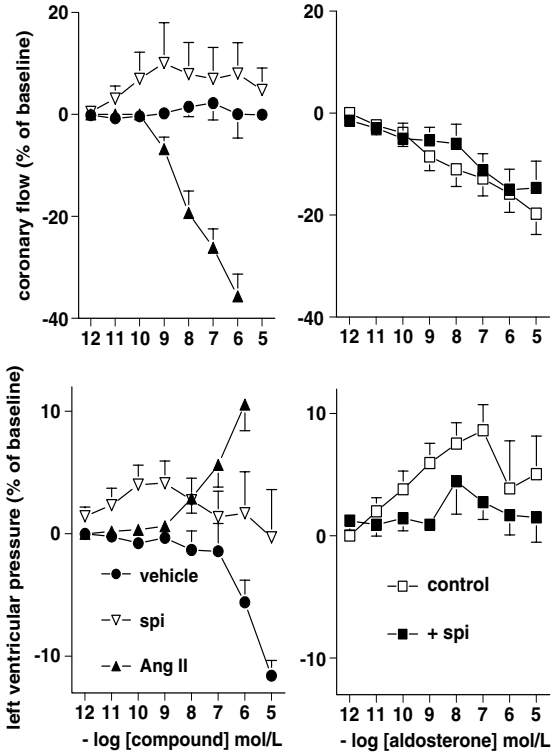

Figure 1 Left panels: Effect of Ang II, spironolactone (spi) and vehicle on CF and LVP. Right panels: Effect of aldosterone on CF and LVP in the absence (control) and presence of 10 μM spironolactone. Values (mean ± s.e.m., $n=6$) are expressed as percentage change from baseline.

Experiments in cells

In cardiomyocytes, aldosterone decreased [^{3}H]thymidine incorporation in a concentration-dependent manner (pEC_{50} 6.6 ± 0.4, $n=9$; Figure 3). Spironolactone ($n=9$) did not affect [^{3}H]thymidine incorporation, and reversed the effects of 1 μmol l^{-1} aldosterone in a concentration-dependent manner (pEC_{50} 6.5 ± 0.3, $n=9$). This indicates that the effects of aldosterone on DNA synthesis depend on MR activation. Ang II (100 nM; $n=7$) increased [^{3}H]thymidine incorporation by 51 ± 6% ($P<0.05$), and aldosterone concentration-dependently inhibited this effect ($n=7$). Vehicle did not affect [^{3}H]thymidine incorporation (100 ± 6%, $n=9$). Total protein (133 ± 13 μg per well) and total DNA (5.3 ± 0.5 μg per well) contents of myocytes were not affected by any of the (ant)agonists, their combination or vehicle (data not shown).

In cardiac fibroblasts, aldosterone decreased [^{3}H]proline incorporation in a concentration-dependent manner (pEC_{50} 7.7 ± 0.7, $n=4$; Figure 3). Spironolactone ($n=4$) did not affect [^{3}H]proline incorporation, and reversed the effects of 1 μmol l^{-1} aldosterone in a concentration-dependent manner

Table 1 Area at risk, fibrillation incidence, and haemodynamic parameters at baseline, after 45 min of coronary artery occlusion ('ischaemia'), and during reperfusion

Parameter			*Pretreatment*		
	None	*Preconditioning*	*Vehicle*	*Aldosterone*	*Spironolactone*
LVEDP (mmHg)					
Baseline	5.2±0.2	5.1±0.2	5.2±0.2	5.3±0.3	5.2±0.2
Ischaemia	9.7±1.3	8.3±1.5	7.8±1.5	7.3±1.0	5.7±0.5
Reperfusion	23±5.3	14±2.0	33±10	24±7.2	11±13
LVP (mmHg)					
Baseline	75±6.9	82±2.3	82±3.1	81±3.7	81±2.7
Ischaemia	27±2.4	28±3.8	30±3.1	33±2.6	34±3.4
Reperfusion	31±3.2	50±1.8*	39±3.5	38±4.9	52±4.9*
CF (ml min^{-1})					
Baseline	13±0.8	13±0.9	13±0.9	11±0.9	12±1.1
Ischaemia	7.7±0.8	6.2±1.5	6.1±0.8	4.0±0.3	6.7±0.7
Reperfusion	10±1.0	11±0.7	10±2.1	7.9±0.5	9.9±0.7
Area at risk (%)	50±3	52±3	52±3	52±3	53±3
Fibrillation incidence	6/6	2/6*	5/6	6/6	2/6*

Data are mean±s.e.m. of six experiments. *$P<0.05$ vs no pretreatment. LVEDP, left ventricular end-diastolic pressure; LVP, left ventricular pressure; CF, coronary flow.

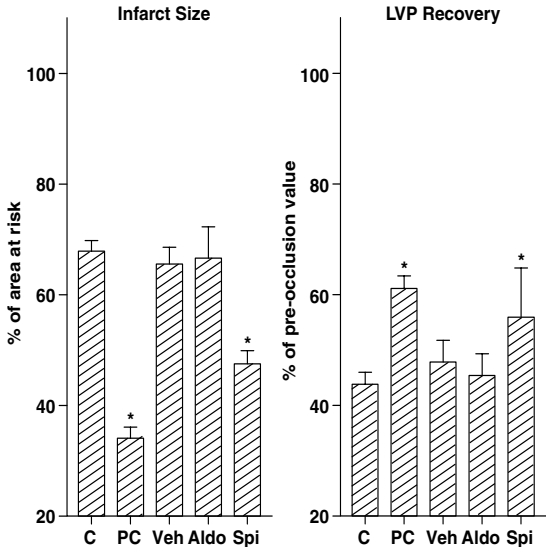

Figure 2 Infarct size (left panel) and recovery of LVP (right panel) in hearts that were subjected to 45 min left anterior descending coronary artery occlusion, followed by 3 h of reperfusion, after either no pretreatment (C, control), preconditioning (PC, 15 min of occlusion followed by 10 min of reperfusion), or a 15-min exposure to vehicle (Veh), 100 nM aldosterone (Aldo), or 100 nM spironolactone (Spi). Values are mean±s.e.m. of six experiments. *$P<0.05$ vs control.

(pEC_{50} 6.6±0.7, $n=4$). This indicates that the effects of aldosterone on collagen synthesis depend on MR activation. Ang II (100 nM; $n=4$) increased [^{3}H]proline incorporation by 33±7% ($P<0.05$), and aldosterone concentration-dependently inhibited this effect ($n=4$). Vehicle did not affect [^{3}H]proline incorporation (98±2%, $n=4$). Total protein (64±14 μg per well) and total DNA (2.1±0.2 μg per well) contents of fibroblasts were not affected by any of the (ant)agonists, their combination or vehicle (data not shown).

In aortic VSMCs, aldosterone decreased [^{3}H]thymidine incorporation in a concentration-dependent manner (pEC_{50} 7.4±0.4, $n=7$; Figure 4). Spironolactone ($n=7$) did not affect [^{3}H]thymidine incorporation, and partially reversed the effects of 1 μmol l^{-1} aldosterone. Ang II (100 nM; $n=7$) did not significantly affect [^{3}H]thymidine incorporation. However, in the presence of 10 nM aldosterone, it increased [^{3}H]thymidine incorporation by 56±15% ($P<0.01$). Higher concentrations of aldosterone decreased [^{3}H]thymidine incorporation in the presence of Ang II, to the same degree as under baseline conditions (Figure 4). Vehicle did not affect [^{3}H]thymidine incorporation (111±22%, $n=7$). Total protein (138±14 μg per well) and total DNA (4.1±0.4 μg per well) contents of VSMCs were not affected by any of the (ant)agonists, their combination or vehicle (data not shown).

Discussion

The present study distinguishes both MR ('genomic')- and non-MR ('nongenomic')-mediated effects of aldosterone in the rat heart. Genomic effects include inhibition of DNA synthesis in cardiomyocytes, inhibition of collagen synthesis in cardiac fibroblasts, and deleterious cardiac effects (including fibrillation) during ischaemia and reperfusion. Nongenomic effects include coronary vasoconstriction and, most likely, positive inotropy. The nongenomic effects of aldosterone on vasoconstriction and inotropy parallel the effects of Ang II on these parameters, whereas the genomic effects on growth and remodelling counteract those of Ang II.

Nongenomic effects

At first sight (Figure 1), it appears that spironolactone blocks the inotropic effects of aldosterone, thus suggesting that these effects are mediated *via* MR activation. However, in contrast with this conclusion, the inotropic effects occurred within minutes, whereas MR-induced effects usually occur after hours. Furthermore, the inotropic effects occurred at the same

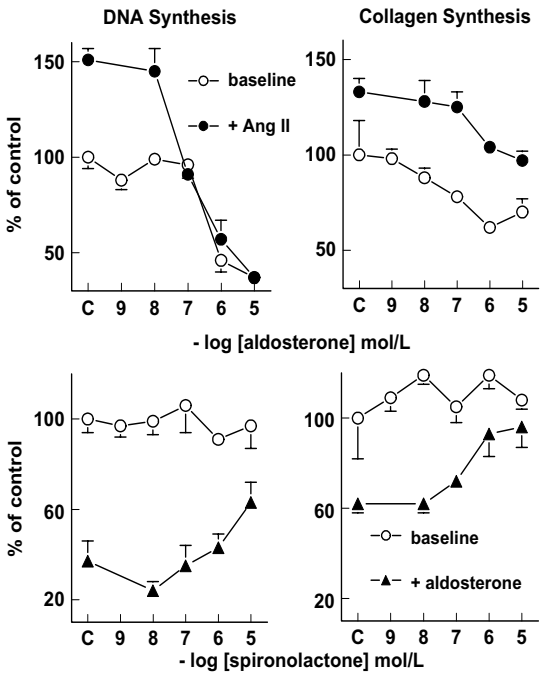

Figure 3 Effect of aldosterone (top panels) and spironolactone (bottom panels) on [^{3}H]thymidine incorporation (DNA synthesis) in myocytes and on [^{3}H]proline incorporation (collagen synthesis) in fibroblasts, either at baseline or in the presence of 100 nM Ang II or 1 μM aldosterone. Values (mean $\pm$ s.e.m., $n = 4$–9) are expressed relative to control; C refers to the response in the absence of aldosterone or spironolactone. Ang II increased [^{3}H]thymidine incorporation by 51 $\pm$ 6% ($P < 0.05$). Aldosterone decreased [^{3}H]thymidine incorporation, both in the presence of Ang II (to 37 $\pm$ 4% of control, $P < 0.001$) and at baseline (to 37 $\pm$ 3% of control, $P < 0.001$). Ang II increased [^{3}H]proline incorporation by 33 $\pm$ 7% ($P < 0.05$), and aldosterone fully reversed this effect, as evidenced by the absence of Ang II-induced effects on collagen synthesis ($P =$ NS vs control) at aldosterone concentrations of 1 μM and higher. At baseline, aldosterone decreased [^{3}H]proline incorporation to 62 $\pm$ 5% ($P < 0.05$) of control. Spironolactone did not exert effects at baseline, and, at a concentration of 10 μM, reversed the aldosterone-induced decreases in DNA and collagen synthesis to levels that were not significantly different from control.

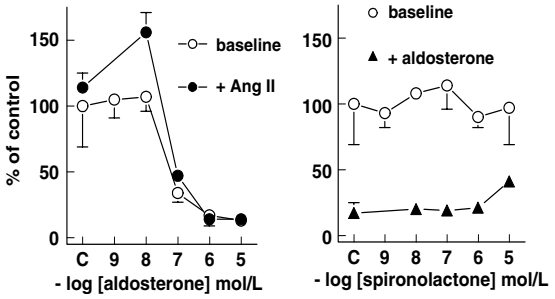

Figure 4 Effect of aldosterone (left panel) and spironolactone (right panel) on [^{3}H]thymidine incorporation in vascular smooth muscle cells, either at baseline or in the presence of 100 nM Ang II or 1 μM aldosterone. Values (mean $\pm$ s.e.m., $n = 7$) are expressed relative to control; C refers to the response in the absence of aldosterone or spironolactone. Ang II did not significantly affect [^{3}H]thymidine incorporation. On top of Ang II, aldosterone exerted a biphasic effect: at a concentration of 10 nM, it increased [^{3}H]thymidine incorporation to 156 $\pm$ 15% of control ($P < 0.01$), and at higher concentrations it decreased [^{3}H]thymidine incorporation to 14 $\pm$ 5% of control ($P < 0.001$). At baseline, aldosterone did not affect [^{3}H]thymidine incorporation at concentrations up to 10 nM, whereas at higher concentrations it decreased [^{3}H]thymidine incorporation to 13 $\pm$ 6% of control ($P < 0.001$). Spironolactone, at a concentration of 10 μM, partially blocked the effects of 1 μM aldosterone, as evidenced by the fact that the aldosterone-induced effects at this spironolactone concentration were still significantly different ($P < 0.05$) from control.

(subnanomolar) aldosterone concentration range as the non-genomic effects on CF, that is, at levels that were approximately 100 times lower than the levels required to induce MR-induced effects in cultured cardiac cells. Finally, spironolactone did not alter aldosterone potency; it only reduced aldosterone efficacy. With regard to the latter, it is important to note that both aldosterone and spironolactone were dissolved in ethanol. Ethanol, like methanol, decreases contractility in a concentration-dependent manner (Tom *et al*., 2001). The spironolactone concentration that we and others (Moreau *et al*., 1996) used (10 μM) corresponds with an ethanol concentration of 1% in the 100 μl bolus injection, and thus the 'blocking' effects of spironolactone may in reality represent ethanol-induced physiological antagonism of aldosterone. Owing to the negative inotropic effect of ethanol, the positive inotropic effect of aldosterone in the present study is probably underestimated. Moreover, when correcting for the ethanol-induced negative inotropic effect, our study would reveal a positive inotropic action for spironolactone as well, thereby further arguing against the MR as the mediator of the inotropic effect of aldosterone. This conclusion is in full agreement with a previous report in the isolated working rat heart, where 10 nM spironolactone increased contractility on top of 10 nM aldosterone (Barbato *et al*., 2002). The ethanol concentration in the perfusion fluid in that study was maximally 0.036%. The ethanol concentration in the perfusion fluid in our experimental setup, at a CF of $\approx 12\,\mathrm{ml\,min^{-1}}$, was most likely $< 1\%$, but $> 0.036\%$. Propanediol has also been used to dissolve aldosterone and spironolactone. This solvent, however, similarly reduces inotropy, and thus the 'blocking' effect of 10 μM spironolactone (dissolved in propanediol) towards 10 nM aldosterone observed in an earlier study in the isolated rat heart (Moreau *et al*., 1996) may well be explained on the basis of the same physiological antagonism described above for ethanol. Taken together therefore, the inotropic effect of aldosterone is unlikely to be mediated *via* MR activation.

Positive inotropy favours coronary vasodilatation, and this may explain why Barbato *et al*. (2002), in the isolated working rat heart, observed vasodilation during a 45% increase in contractility in response to 10 nM aldosterone, as opposed to the aldosterone-induced vasoconstriction observed in the present study. In our Langendorff setup, the inotropic response to aldosterone was of modest proportion (maximally $\approx 10\%$) and this may not have been sufficient to result in a rise in CF. Rapid nongenomic vasoconstrictor effects of aldosterone have been described before (Schmidt *et al*., 2003), and hyperaldosteronism in hypertensive subjects results in impaired endothelium-dependent flow-mediated vasodilation (Nishizaka *et al*., 2004).

Ang II, like aldosterone, and in agreement with previous studies (de Lannoy *et al*., 1997; 1998; Libonati *et al*., 1997; Müller *et al*., 1998) caused coronary vasoconstriction and increased inotropy. The effects of Ang II were larger, occurred later and lasted longer than the effects of aldosterone on these

parameters. Owing to this difference in time course (which has been noted before in cultured cells (Mazak *et al*., 2004)), we did not attempt to study the combined effects of Ang II and aldosterone on coronary vasoconstriction and inotropy. The difference in time course suggests that separate mechanisms underlie the contractile responses to Ang II and aldosterone, so that additive effects and/or synergy might be expected following combined application (Mazak *et al*., 2004; Michel *et al*., 2004).

Genomic effects

Spironolactone significantly improved the condition of the heart following ischaemia and reperfusion, as evidenced by a reduction in infarct size, incidence of fibrillation and LVEDP, and an increase in LVP recovery. The protective effects of spironolactone were comparable to those of preconditioning (Schoemaker & van Heijningen, 2000), and they cannot be contributed to ethanol, as in this part of the study we used a spironolactone concentration of 100 nM, that is, a concentration that is sufficiently low to avoid the ethanol-induced negative inotropic effects. Chronic *in vivo* treatment of rats with spironolactone similarly improved the condition of the heart when it was mounted in the Langendorff apparatus and exposed to low-flow ischaemia (Rochetaing *et al*., 2003). However, under those conditions it could not be concluded to what extent the effects of spironolactone were of cardiac origin. Our data now provide evidence that the beneficial effects of MR blockade during ischaemia indeed originate in the heart. There are several explanations for this finding. First, spironolactone may prevent adverse MR-mediated effects of locally synthesized aldosterone. Such effects include proarrhythmogenic actions (Tillmann *et al*., 2002) and increased synthesis of oxygen radicals (Mazak *et al*., 2004). Second, spironolactone might exert beneficial effects of its own, independently of aldosterone, for example, it acts antiarrhythmogenic through blockade of human Ether-a-Go-Go-Related gene (HERG) K^+ channels (Caballero *et al*., 2003), and it inhibits calcium entry through calcium channels (Cargnelli *et al*., 2001). The lack of additional deleterious effects of aldosterone exposure, in addition to the evidence for aldosterone synthesis in the rat heart (Silvestre *et al*., 1998), particularly under ischemic conditions (Silvestre *et al*., 1999), favours the concept that spironolactone interferes with locally released aldosterone.

Aldosterone inhibited DNA and collagen synthesis in cultured cardiac cells, and spironolactone blocked these effects, thereby providing evidence for their MR-dependency. The aldosterone concentrations required to inhibit DNA and collagen synthesis were $\approx$100 times higher than the concentrations that induced vasoconstriction and positive inotropy. On the one hand, this suggests that the effects in cultured cells are indeed mediated *via* mechanisms/receptors different from the constrictor and inotropic effects in the intact heart, whereas, on the other hand, it is questionable whether such high concentrations actually exist at tissue sites *in vivo*. High aldosterone concentrations are required to activate cardiac MRs, because the levels of 11β-hydroxysteroid dehydrogenase 2 in the heart are relatively low (Lombès *et al*., 1995). This enzyme converts the MR-binding glucocorticoids cortisol and corticosterone (which circulate at concentrations that are several orders of magnitude higher than those of aldosterone) into the their non-MR-binding metabolites cortisone and 11-dehydrocorticosterone. Normally, glucocorticoid binding to MRs in cells maintained under serum-free conditions should not occur, unless the receptors are still occupied by glucocorticoids bound to the cells during their incubation with serum. Such cell binding also underlies the presence of renin–Ang system components in cardiomyocytes under serum-free conditions (van Kesteren *et al*., 1999).

The inhibitory effects of aldosterone on cardiac growth and remodelling contrast with *in vivo* studies showing cardiac fibrosis following long-term aldosterone exposure (Sun *et al*., 1993; 2002). However, fibrosis is also observed following conditional expression of an antisense mRNA of the MR in the mouse heart (Beggah *et al*., 2002). Thus, the effect of circulating aldosterone may differ from the effect of locally synthesised aldosterone, and myocardial fibrosis is unlikely to be a direct effect of aldosterone on cardiac fibroblasts. The effects of aldosterone on DNA and collagen synthesis oppose the effects of Ang II on these parameters, and this may explain why aldosterone, on top of Ang II, exerts a modulatory role in the development of cardiac hypertrophy in humans (Osterop *et al*., 1998; Deinum *et al*., 2001; Tsybouleva *et al*., 2004).

Finally, we verified the response of aldosterone on DNA synthesis in aortic VSMCs. When given at baseline, aldosterone reduced [^{3}H]thymidine incorporation, and this effect was blocked, at least in part, by spironolactone. Thus, as in cardiomyocytes, aldosterone inhibited DNA synthesis in an MR-dependent manner. The fact that spironolactone did not completely block the effect of aldosterone suggests that other (nongenomic) pathways may also have played a role. Alternatively, the highest spironolactone concentration in this study (10 μM) may have been insufficient to fully prevent the almost complete inhibition of DNA synthesis in the presence of 1 μM aldosterone in VSMCs. In cardiomyocytes, the prevention at this concentration of spironolactone was more complete (allowing DNA synthesis to return to levels that were not significantly different from control), possibly because the effect of aldosterone in these cells was of more modest proportion.

In the presence of Ang II, aldosterone exerted a biphasic effect on DNA synthesis in VSMCs: at low concentrations (nM range) it enhanced the response to Ang II, and at high concentrations (μM range) it blocked the response to Ang II. Such a biphasic, Ang II-dependent, [^{3}H]thymidine incorporation response to aldosterone has been noted before in rat VSMCs (Xiao *et al*., 2004). The mechanism mediating the enhanced response is not known, but may involve upregulation of AT_1 receptors (Xiao *et al*., 2004). The fact that it occurred at nM concentrations of aldosterone is suggestive for a nongenomic phenomenon.

Conclusion

Aldosterone induces positive inotropic and vasoconstrictor effects in a nongenomic manner, and may thus potentiate the effects of Ang II on inotropy and vasoconstriction. Aldosterone reduces DNA and collagen synthesis *via* MR activation, and antagonises the Ang II-induced increases in these parameters. MR blockade reduces infarct size and restores cardiac function following a 45-min period of ischaemia to a similar degree as preconditioning. The cardiac MR-mediated effects may help to explain, at least in part, the beneficial actions of spironolactone and other MR antagonists on top of ACE inhibition in heart failure (Pitt *et al*., 1999; 2003).

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Chapter 5

Cardioprotective effects of eplerenone in the rat heart: interaction with locally synthesized or blood-derived aldosterone?

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Submitted

Summary

Mineralocorticoid receptor (MR) antagonism with eplerenone reduces mortality in heart failure, possibly due to blockade of the deleterious effects of cardiac aldosterone. To investigate these effects, rat Langendorff hearts were exposed to aldosterone and/or eplerenone under normal and ischemic conditions. Under normal conditions, aldosterone increased left ventricular pressure (LVP) and decreased coronary flow. Eplerenone did not block these effects. Eplerenone reduced infarct size (from 68±2 to 53±4%, $P<0.05$) and increased LVP recovery (from 44±2% to 60±5%, $P<0.05$) after 45 minutes of coronary artery occlusion and 3 hours of reperfusion, whereas aldosterone did not affect these parameters. To verify the origin of cardiac aldosterone, hearts were perfused with 3-30 nmol/L aldosterone, and either frozen immediately or exposed to washout. Without washout, cardiac aldosterone was 1.5 times aldosterone in coronary effluent (CE), i.e. too high to be explained on the basis of its presence in extracellular fluid. The cardiac levels of aldosterone correlated with its CE levels ($r=0.81$, $P<0.01$), and both were unaffected by eplerenone. During washout, tissue aldosterone disappeared monophasically ($t_{½}$ 9±1 min), and CE aldosterone disappeared biphasically ($t_{½}$'s 1±0 and 8±1 min, respectively). During buffer perfusion, cardiac aldosterone was at or below the detection limit. In conclusion, eplerenone improves the condition of the heart following ischemia and reperfusion. This does not relate to interference with the inotropic and vasoconstrictor effects of aldosterone. The majority of cardiac aldosterone, if not all, is derived from the circulation. The rapid, MR-independent, kinetics of aldosterone suggests that its accumulation in the heart involves cell surface binding rather than internalization.

Introduction

Two large clinical trials in patients with heart failure have recently shown that the mineralocorticoid receptor (MR) antagonists spironolactone and eplerenone improve morbidity and mortality on top of ACE inhibition [1,2]. In particular, a reduction in the rate of sudden death was observed. The mechanism responsible for this favorable effect is not entirely understood. It may involve changes in Na^+/K^+ homeostasis and/or myocardial fibrosis inhibition. In addition, conditional MR overexpression in the mouse heart, in the absence of aldosteronemia, was recently shown to result in severe arrhythmias [3]. Thus, cardiac MR may also trigger arrhythmias directly.

In support of this possibility, spironolactone improved electrophysiological parameters in subjects with heart failure [4], and, in combination with the ACE inhibitor fosinopril, reduced the arrhythmic score post-myocardial infarction [5]. Since spironolactone is a non-specific MR antagonist, these effects do not necessarily involve MR [6]. Spironolactone also reduced infarct size and improved the recovery of left ventricular pressure following 45 minutes of global ischemia in the isolated perfused rat heart [7]. Interestingly, spironolactone did not block aldosterone-induced vasoconstriction, which worsens cardiac contractile and metabolic function in the ischemic heart [8,9]. It also did not prevent the inotropic effects of aldosterone in the heart [7,10], and, if anything, exerted inotropic effects of its own, independently of aldosterone.

The aldosterone concentrations required to exert the above inotropic and vasoconstrictor effects are in the nanomolar range [7,9,10]. In the heart, it has been proposed that the presence of such high levels depend on local synthesis of aldosterone, although not all studies agree on this issue [11-13]. Across the human coronary vascular bed, both release and uptake of aldosterone have been observed [14,15].

In the present study, using the isolated perfused rat Langendorff heart, we first investigated whether the new and more selective MR antagonist eplerenone exerts the same cardioprotective effects as spironolactone, in order to further unravel whether these effects are MR-mediated. Secondly, we studied the cardiac uptake and washout of circulating aldosterone, to determine whether eplerenone, if exerting a MR-dependent effect, interferes with locally synthesized or blood-derived aldosterone.

Methods

Drugs

Aldosterone was purchased from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands. Eplerenone was a kind gift of Pfizer, Capelle a/d IJssel, The Netherlands. Stock solutions of aldosterone (10 mmol/L) and eplerenone (10 mmol/L) were prepared in ethanol.

Ethical Approval

All experiments were performed under the regulations of the Animal Care Committee of the Erasmus MC, in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the American Physiological Society.

Experiments in Langendorff Hearts

Male Wistar rats (n=75, weight 300-420 g), obtained from Harlan, Zeist, The Netherlands, were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.). Hearts were rapidly excised and cooled in iced-cold Krebs-Henseleit solution (composition in mmol/L: NaCl 125, KCl 4.7, $NaHCO_3$ 20, NaH_2PO_4 0.43, $MgCl_2$ 1.0, $CaCl_2$ 1.3 and D-glucose 9.1; pH 7.4) until contractions stopped, and prepared for Langendorff perfusion. Continuously carbogen-gassed (95% O_2 / 5% CO_2) Krebs-Henseleit solution at 37°C was perfused immediately after cannulation of the aorta, at a constant perfusion pressure of 80 mmHg. A water-filled latex balloon was placed in the left ventricle *via* the left atrium to measure LVP. The volume of the balloon was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) of 5 mm Hg during initial equilibration, and this volume was maintained throughout the experiment. Hearts were paced at 350 beats/minute. CF was measured by an in-line flow probe (Transonic Systems, Ithaca, NY, USA).

After a stabilization period of 15 minutes, 100 μL bolus injections were applied to construct dose-response curves to aldosterone, vehicle (ethanol) and/or eplerenone. In a second series of experiments, we evaluated the effects of aldosterone and eplerenone

during ischaemia and reperfusion. Hearts were subjected to 45 minutes left anterior descending coronary artery occlusion, followed by 3 hours of reperfusion. Occlusion was preceded by either no treatment (control) or a 15-minute exposure 100 nmol/L aldosterone, 1 μmol/L eplerenone or 100 nmol/L aldosterone + 1 μmol/L eplerenone. Aldosterone and/or eplerenone remained present in the perfusion buffer throughout the remainder of the experiment. After the 3-hour reperfusion period, area at risk and infarct size were determined as described before. In a third series of experiments, we determined the uptake and disappearance of aldosterone in the heart. Blood (≈0.5 mL) was collected from the rats used in these experiments to measure the endogenous plasma levels of aldosterone. Hearts were perfused with 3, 10 or 30 nmol/L aldosterone or vehicle for 30 minutes, in the absence or presence of 1 μmol/L eplerenone. After the 30-minute perfusion period, the hearts were either frozen in liquid nitrogen or subjected to a washout period. One-minute samples of coronary effluent were collected prior to the perfusion, at 20 and 25 minutes after the start of the perfusion, and at 1, 2, 3, 4, 5, 10, 20 and 30 minutes during the washout period. Aldosterone-perfused hearts that had been washed for 5 or 30 minutes were also frozen in liquid nitrogen.

Aldosterone Measurements

Aldosterone was measured by solid-phase radioimmunoassay (DPC) in rat plasma, coronary effluent and cardiac tissue. To extract aldosterone from cardiac tissue, hearts were homogenized 1:2 in methanol. The homogenate was centrifuged at 3,000 rpm for 15 minutes. Supernatants were then collected, vacuum dried, and dissolved in water prior to the assay. The detection limit was 25 pg/mL in plasma and coronary effluent, and 10 pg/g wet weight.

Data analysis

Data are expressed as mean±SEM. Dose-response curves were analyzed as described before[16] to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. Statistical analysis was by ANOVA, followed by post hoc evaluation according to Tukey. P values <0.05 were considered significant.

Results

Haemodynamic studies

Baseline values of LVP and CF were 80±1.6 mm Hg (n=66) and 11±0.2 mL/minute, respectively. Aldosterone (n=6) dose-dependently increased LVP (pEC_{50} 9.8±0.4) and reduced CF (pEC_{50} 8.7±0.6) (Figure 1). Eplerenone (n=6) also increased LVP (pEC_{50} 8.9±1.3) without affecting CF (Figure 1). Vehicle (ethanol; n=6) did not affect LVP or CF. Eplerenone (0.1 or 1 μmol/L, n=6 and 5, respectively) did not abolish the effects of aldosterone on LVP or CF.

Occlusion of the left anterior descending coronary artery reduced LVP to ≈30 mm Hg and decreased CF by approximately 50% (Table 1). In control hearts (n=6), LVP and CF increased rapidy during the reperfusion phase (Table 1), stabilizing after approximately 20 minutes at 44±3% and 85±13% of pre-ischaemia values (Figure 2). LVEDP increased to 23±5 mm Hg. Pretreatment with aldosterone (n=6) did not affect LVP recovery, nor did it prevent the rise in LVEDP. Aldosterone tended to reduce CF recovery (P=NS, Figure 2). Pretreatment with eplerenone (n=6) significantly enhanced recovery of LVP ($P<0.05$), but not of CF (Table 1 and Figure 2). Eplerenone did not diminish the rise in LVEDP. Ventricular fibrillation occurred within the first 10 minutes of reperfusion of all control hearts (Table 1). Eplerenone, but not aldosterone, tended to reduce the incidence of fibrillation (P=NS). The area at risk was identical in all hearts (Table 1). Infarct size (expressed as a percentage of the area at risk) was 68±2% in control hearts (Figure 2). Eplerenone reduced infarct size ($P<0.05$), whereas the infarct size in aldosterone-treated hearts was not different from control. Combined exposure to eplerenone and aldosterone yielded similar results as eplerenone alone (Table 1, Figure 2).

Table 1 *Haemodynamic parameters, area at risk, and fibrillation incidence at baseline, after 45 minutes of coronary artery occlusion ('ischaemia'), and during reperfusion.*

Parameter	**Pretreatment**			
	None	**Aldo**	**Eple**	**Aldo+Eple**
Left ventricular end-diastolic pressure, mm Hg				
baseline	5.2±0.2	5.3±0.3	5.3±0.3	5.5±0.9
ischaemia	9.7±1.3	7.3±1.0	7.3±1.0	6.7±1.5
reperfusion	23±5.3	24±7.2	24±7.2	23±2.8
Left ventricular pressure, mm Hg				
baseline	75±6.9	81±3.7	85±6.7	84±5.9
ischaemia	27±2.4	33±2.6	36±3.3	36±3.0
reperfusion	31±3.2	38±4.9	50±3.1*	49±3.5*
Coronary flow, ml/minute				
baseline	13±0.8	11±0.9	12±0.9	12±0.7
ischaemia	7.7±0.8	4.0±0.3	7.8±0.8	4.8±0.5
reperfusion	10±1.0	7.9±0.5	11±1.3	11±1.6
Area at risk, %	50±3	52±3	49±2	50±3
Fibrillation incidence	6/6	6/6	4/6	5/7

*Data are mean±SEM of 6-7 experiments. *, P<0.05 vs. no pretreatment. Aldo, aldosterone; Eple, eplerenone.*

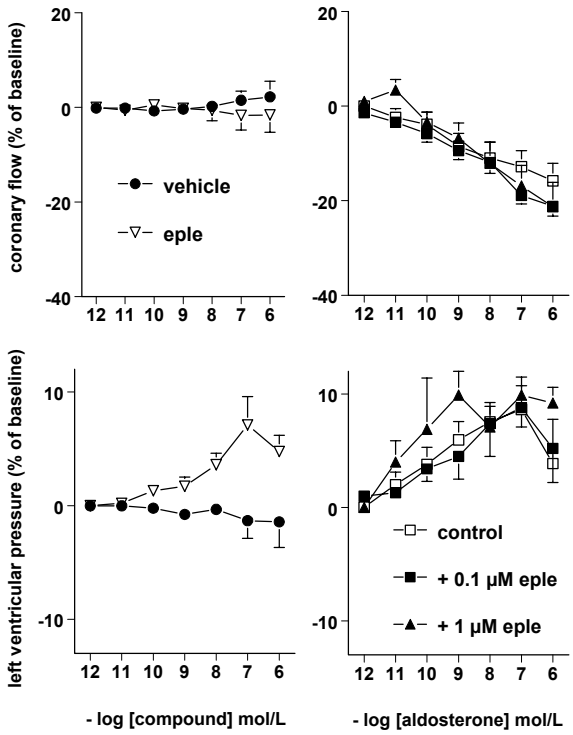

Figure 1 Left panels. *Effect of eplerenone (eple) and vehicle on coronary flow and left ventricular pressure.* Right panels. *Effect of aldosterone on coronary flow and left ventricular pressure in the absence (control) or presence of 0.1 or 1 µmol/L eplerenone. Values (mean±SEM, n=6) are expressed as percentage change from baseline.*

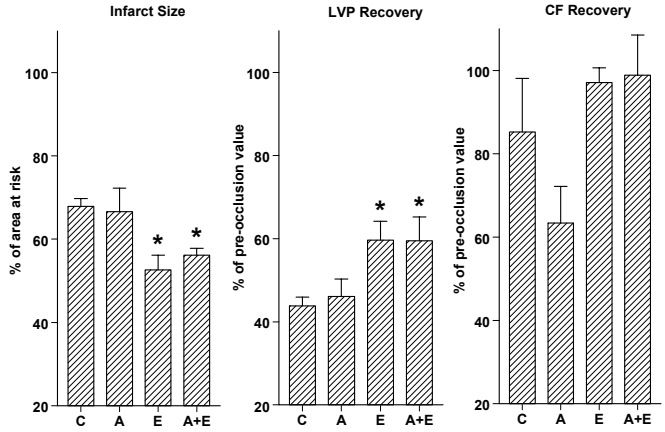

Figure 2 *Infarct size (left panel), recovery of left ventricular pressure (LVP) (middle panel), and recovery of coronary flow (CF) in hearts that were subjected to 45 minutes left anterior descending coronary artery occlusion, followed by 3 hours of reperfusion, after either no pretreatment (C, control) or a 15-minute exposure to 100 nmol/L aldosterone (A), 1 µmol/L eplerenone (E) or 100 nmol/L aldosterone + 1 µmol/L eplerenone. Values are mean±SEM of 6-7 experiments. * P<0.05 vs. control.*

Kinetic studies

Aldosterone levels in rat plasma were 85±16 pg/mL (n=5). Aldosterone levels in the perfusate of vehicle-perfused hearts were <58±15 pg/mL. In 5 out of 8 hearts these levels were below the detection limit. The cardiac aldosterone levels in vehicle-perfused hearts were <20±5 pg/g wet weight. In 1 out of 3 hearts this level was below the detection limit. The level of aldosterone in the coronary effluent during perfusion with 10 nmol/L aldosterone was 2161±87 pg/mL at 20 minutes, and 2132±99 pg/mL at 25 minutes (p=NS, n=9), indicating that a steady state had been reached. The steady-state level in coronary effluent was 97±5% of the level in the perfusion buffer. After discontinuation of the aldosterone perfusion, aldosterone disappeared from the coronary effluent in a biphasic manner (n=6, Figure 3). The rapid phase had a t½ of 1.1±0.1 minute, and the slow phase had a t½ of 7.9±1.1 minutes. The cardiac tissue level of aldosterone, immediately after the 10 nmol/L aldosterone perfusion had been switched off, was 2533±327 pg/g wet weight (n=3). During the washout phase, the tissue level decreased in a monophasic way, with a t½ of 9.1±1.0 minutes (Figure 3). Eplerenone, at a concentration of 1 μmol/L, did not affect the steady-state tissue level reached following a 30-minute perfusion with 10 nmol/L aldosterone (3471±197 pg/g wet weight, n=3; P=NS vs. without eplerenone). The cardiac tissue levels after a 30-minute perfusion with 3 or 30 nmol/L aldosterone were 982±207 and 6720±941 pg/g wet weight (n=3 for both), respectively. Tissue levels (expressed per gram wet weight) closely correlated with coronary effluent levels (expressed per mL effluent) (r=0.81, P<0.01; Figure 4).

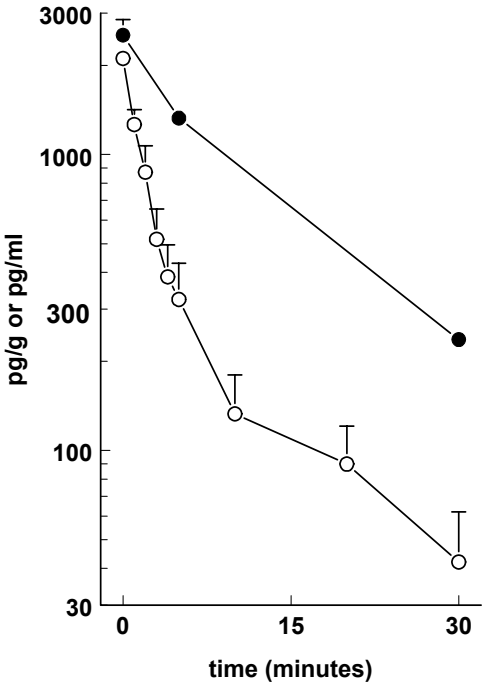

Figure 3 *Washout of aldosterone from coronary effluent (open circles) and cardiac tissue (closed circles) of the Langendorff heart after its perfusion with 10 nmol/L aldosterone for 30 minutes. Values are mean±SEM of 3-6 experiments.*

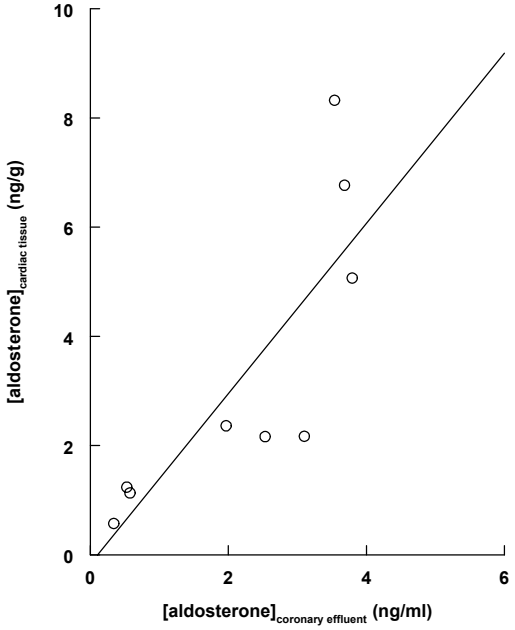

Figure 4 *Correlation between the levels of aldosterone in coronary effluent and cardiac tissue (r=0.81, P<0.01) following a 30-minute perfusion of the heart with 3, 10 or 30 nmol/L aldosterone.*

Discussion

The present study reveals no blocking effect of eplerenone towards the inotropic and vasoconstrictor effects of aldosterone in the rat Langendorff heart, thereby confirming that these effects are exerted in a 'non-genomic' manner, not involving MR [7,10]. In fact, eplerenone, like spironolactone, induced a modest inotropic effect of its own. The eplerenone concentration required to induce this inotropic effect was ≈3 orders of magnitude higher than the spironolactone concentration required to induce a similar effect [7,10], and thus, since the doses of both drugs are comparable, inotropic effects are less likely to occur during treatment with eplerenone than during treatment with spironolactone. Unlike spironolactone, eplerenone did not affect coronary flow. Possibly therefore, the spironolactone-induced effects on flow are mediated through a different receptor than its effects on contractility. The latter has been reported to involve increased myosin ATPase calcium sensitivity and diastolic calcium concentration [17].

Eplerenone protected the heart during ischaemia and reperfusion in a similar way as spironolactone (inducing a reduction in infarct size and improving LVP recovery), and therefore, this cardioprotective effect is most likely MR-mediated. It could relate to blockade of the proarrhythmogenic actions of aldosterone [3,18] and/or the aldosterone-induced increase in oxygen radical synthesis [19]. If so, aldosterone was apparently still present in the isolated perfused rat Langendorff heart. It does not relate to the non-MR-mediated coronary constrictor effects of aldosterone [8,9].

Aldosterone, at a concentration of 100 pmol/mL, did not further deteriorate the condition of the heart during the ischaemia + reperfusion procedure. Thus, the endogenous aldosterone levels in the heart were presumably <100 pmol/g, despite earlier reports by Silvestre et al. [11,20] describing such high aldosterone levels in the isolated perfused rat heart (50-500 pg/mg protein or, since 1 gram of tissue corresponds with 65-100 mg protein [13,21], ≈10-150 pmol/g). Indeed, the cardiac tissue levels of aldosterone in our study were <20 pg/g. In some of our experiments, aldosterone was detectable in coronary effluent, obtained during the 30 minutes of perfusion with vehicle prior to the collection of the heart. Although this could reflect local synthesis of aldosterone [12], it

might also represent washout of blood-derived aldosterone that had accumulated in the heart in vivo.

To study the kinetics of uptake and washout of circulating aldosterone in the heart, we perfused the rat Langendorff heart with aldosterone, using concentrations corresponding with those in patients with severe heart failure [9,14,15]. Aldosterone perfusion resulted in a rapid rise of the cardiac aldosterone levels, independently of MR. After 30 minutes the tissue levels (expressed per g wet weight) were on average 1.5 times higher than the levels in coronary effluent (expressed per mL). Since the extracellular volume in this preparation is around 0.6 mL/g [22,23], the cardiac aldosterone levels are 2-3 times higher than expected if the presence of aldosterone was limited to the extracellular fluid. Apparently therefore, aldosterone accumulates in a compartment other than extracellular fluid, e.g. it binds to cell surface receptors and/or reaches intracellular sites. In support of this concept, the washout of aldosterone after stopping the perfusion followed a biphasic pattern: a rapid phase corresponding with its disappearance from extracellular fluid [22,24], and a slow phase corresponding with its washout from a second compartment. This pattern resembles that of cardiac renin, which accumulates in extracellular fluid and binds to membrane receptors [22,25,26].

Following perfusion of the Langendorff heart with aldosterone, the cardiac aldosterone levels correlated with the aldosterone levels in the perfusion buffer over a wide range, indicating a large capacity of the heart to accumulate aldosterone. Extrapolating this relationship to the plasma aldosterone levels in the rat (84 pg/mL) yields an in vivo cardiac tissue level of around 1.5 x 84 ≈ 125 pg/g, in full agreement with the cardiac tissue levels reported by others in rats on a normal salt diet [13,27]. These levels are >6 times the level present after buffer perfusion. Apparently, the 15 minutes of equilibration + 30 minutes of perfusion with aldosterone-free buffer prior to the collection of the heart were sufficient to wash away >85% of cardiac aldosterone. This is exactly what one would expect on the basis of a t½ of ≈8-9 minutes for the aldosterone disappearance from tissue sites, and thus it can be concluded that the majority of cardiac aldosterone, if not all, is taken up from the circulation.

Perspective

The present study complements previous studies on the disappearance of cardiac aldosterone following adrenalectomy [13,27] and simultaneously provides an explanation for the reduction in sudden death among patients taking MR antagonists [1,2]. On the one hand, the heart displays a large capacity to accumulate aldosterone. This explains why cardiac aldosterone in rats overexpressing human renin and human angiotensinogen (>99% of which is derived from the adrenal gland) can be up to 10-fold higher than in serum [27]. On the other hand, cardiac aldosterone disappears rapidly during perfusion with aldosterone-free buffer. This provides an explanation for the presence of aldosterone in the effluent of buffer-perfused hearts [12]. The rapid, MR-independent, kinetics of aldosterone observed in this study suggests that its cardiac accumulation involves cell surface binding rather than internalization followed by binding to intracellular MR. The cardioprotective effects of eplerenone during ischemia and reperfusion in the Langendorff preparation are most likely due to blockade of the MR-mediated arrhythmogenic effects [3] of this cell surface-bound aldosterone.

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Chapter 6

Nongenomic effects of aldosterone in the human heart Interaction with angiotensin II

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Nongenomic Effects of Aldosterone in the Human Heart
Interaction With Angiotensin II

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Abstract—Aldosterone exerts rapid "nongenomic" effects in various nonrenal tissues. Here, we investigated whether such effects occur in the human heart. Trabeculae and coronary arteries obtained from 57 heart valve donors (25 males; 32 females; 17 to 66 years of age) were mounted in organ baths. Aldosterone decreased contractility in atrial and ventricular trabeculae by maximally 34±3% and 15±4%, respectively, within 5 to 15 minutes after its application. The protein kinase C (PKC) inhibitor chelerythrine chloride, but not the mineralocorticoid receptor antagonists spironolactone and eplerenone, blocked this effect. Aldosterone also relaxed trabeculae that were prestimulated with angiotensin II (Ang II), and its negative inotropic effects were mimicked by hydrocortisone (at 10-fold lower potency) but not 17β-estradiol. Aldosterone concentrations required to reduce inotropy were present in failing but not in normal human hearts. Previous exposure of coronary arteries to 1 μmol/L aldosterone or 17β-estradiol (but not hydrocortisone) doubled the maximum contractile response (E_{max}) to Ang II. ΔE_{max} correlated with extracellular signal-regulated kinase (ERK) 1/2 phosphorylation ($P<0.01$). Spironolactone and eplerenone did not block the potentiating effect of aldosterone. Studies in porcine renal arteries showed that potentiation also occurred at pmol/L aldosterone levels but not at 17β-estradiol levels <1 μmol/L. Aldosterone did not potentiate the α_1-adrenoceptor agonist phenylephrine. In conclusion, aldosterone induces a negative inotropic response in human trabeculae (thereby antagonizing the positive inotropic actions of Ang II) and potentiates the vasoconstrictor effect of Ang II in coronary arteries. These effects are specific and involve PKC and ERK 1/2, respectively. Furthermore, they occur in a nongenomic manner, and require pathological aldosterone concentrations. **(*Hypertension*. 2005;46:701-706.)**

Key Words: aldosterone ■ mineralocorticoids ■ angiotensin ■ human

The steroid hormone aldosterone is synthesized in the adrenal cortex in response to angiotensin II (Ang II). The primary cardiovascular effect of aldosterone has traditionally been ascribed to regulation of electrolyte homeostasis and extracellular fluid volume by promotion of sodium retention and potassium excretion in the renal collecting duct. The mechanism underlying this effect is of "genomic" nature (ie, it involves binding to the intracellular mineralocorticoid receptor [MR], followed by translocation of the steroid–receptor complex to the nucleus, where this complex acts as a transcription factor).

Two large clinical trials in patients with heart failure have recently shown that MR antagonists improve morbidity and mortality on top of angiotensin-converting enzyme inhibition.[1,2] Because these beneficial effects could not be attributed solely to blockade of the renal MR-mediated effects on blood pressure, it has been proposed that aldosterone also exerts actions in extrarenal tissues. Evidence for this concept is now readily available. The nonrenal actions of aldosterone include inotropic effects in the heart and vasoconstrictor as well as vasodilator effects in various vascular beds.[3–7] Unexpectedly, these effects occurred within minutes rather than hours and could not always be blocked by MR antagonists. Consequently, they are now known as "nongenomic" effects of aldosterone. Some of these nongenomic effects might in fact be exerted by aldosterone that has been synthesized locally in the heart or vessel wall, although not all studies agree on this possibility.[8–10]

The majority of the data on the nongenomic actions of aldosterone have been obtained in animals. It was the aim of the present study to investigate the nongenomic effects of aldosterone in the human heart, focusing on trabeculae and coronary arteries. To determine the specificity of the effects of aldosterone, we also assessed the effects of 17β-estradiol and hydrocortisone. Furthermore, because aldosterone exerts its effects, at least in part, via Ang II or Ang II type 1 (AT_1) receptors,[7,11] and vice versa,[10] we evaluated the interaction with Ang II in our experimental setup. Finally, we looked into the mediators of the effects of aldosterone in this study, focusing on those that have already been coupled to the nongenomic actions of aldosterone (protein kinase C [PKC], Ca^{2+}, NO, and mitogen-activated

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protein kinases),[3,6,7,10,12] and we determined the aldosterone levels in normal and failing human hearts to put our findings into a physiological perspective.

Methods

Tissue Collection

All studies were approved by the ethics committee of the Erasmus MC. Human trabeculae, human coronary arteries (HCAs), and left ventricular tissue were obtained from 57 heart-beating organ donors (25 men and 32 women; 46±1 years of age [range 17 to 66]) who died of noncardiac causes (46 cerebrovascular accident, 5 head trauma, and 6 brain hypoxia) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves. Hearts were stored in an ice-cold sterile organ-protecting solution after circulatory arrest. After arrival at the laboratory, right atrial and left ventricular trabeculae of ≈1-mm thickness were dissected and mounted in organ baths.[13] The trabeculae were paced at 1 Hz using electrical field stimulation. Resting tension was set at 750 mg and 1950 mg for atrial and ventricular trabeculae, respectively. Changes in contraction were recorded with a force transducer. HCAs were removed and stored overnight at 4°C, cut into segments of ≈4-mm length, and suspended on stainless steel hooks in organ baths.[13] Resting tension was set at 1.5 g. Left ventricular tissue pieces (5 to 10 g) were dissected from the heart and frozen at −70°C. Left ventricular tissue (3 to 5 g) was also obtained from 11 subjects (10 men and 1 woman; 48±4 years of age [range 30 to 64]) with end-stage dilated cardiomyopathy undergoing cardiac transplantation.[14]

Porcine renal arteries (PRAs) were removed from kidneys obtained at the slaughterhouse. Vessels were stored overnight and suspended in organ baths as described above. Resting tension was set at 2 g.

Trabeculae Studies

Trabeculae were allowed to equilibrate for ≥60 minutes, and organ bath fluid was refreshed every 15 minutes during this period. Next, a concentration-response curve (CRC) to norepinephrine was constructed to verify the viability of the tissue. After several washouts and stabilization at baseline contractile force, CRCs to aldosterone, spironolactone, eplerenone (a gift of Pfizer), hydrocortisone, their solvent (ethanol), or 17β-estradiol (dissolved in water) were constructed, either at baseline or after prestimulation with forskolin (dissolved in dimethylsulfoxide [DMSO]) or Ang II. To investigate the mechanism of the aldosterone-induced effects, CRCs to aldosterone were also constructed after preincubation with spironolactone, eplerenone, the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), the NO scavenger hydroxocobalamin (dissolved in methanol), the guanylyl cyclase inhibitor ODQ, the PKC inhibitor chelerythrine chloride (dissolved in DMSO), the intracellular Ca^{2+} blocker thapsigargin (dissolved in ethanol), or solvent. At the end of each experiment, viability was verified with norepinephrine.

Vessel Studies

Viability was evaluated as described previously.[13] Next, segments were preincubated for 30 minutes with or without aldosterone, 17β-estradiol, spironolactone, eplerenone, or L-NAME, and CRCs to Ang II, aldosterone, hydrocortisone, 17β-estradiol, solvent (ethanol), or phenylephrine were constructed. Ang II CRCs were also constructed after completion of the CRCs to aldosterone, hydrocortisone, 17β-estradiol, or solvent (ethanol) without refreshing the organ bath fluid in between. To determine Ang II–induced extracellular signal-regulated kinase (ERK) 1/2 activation, segments were collected and frozen at −80°C within 10 minutes after exposure to the highest Ang II concentration.

Biochemical Measurements

To determine ERK 1/2 phosphorylation, frozen HCA segments were homogenized in Nonidet P-40 lysis buffer and kept on ice for 1 hour. Next, they were centrifuged at 14 000*g* for 15 minutes at 4°C, and the supernatants were collected and stored at −80°C until further analysis. Protein was determined using the Bradford assay. Western blotting was performed with 20 μg protein using phospho-ERK 1/2 and ERK antibodies (Westburg; 1:2000). Peroxidase-conjugated secondary antibodies were from Pierce (1:5000). Blots were developed with the chemiluminescence substrate and visualized on Kodak films. For semiquantification, the band obtained with Ang II alone was defined as 100%.

Aldosterone was measured by solid-phase radioimmunoassay (Diagnostic Products Corporation) after its extraction from left ventricular tissue. In short, tissue was homogenized 1:2 in methanol. The supernatant was collected after a 15-minute centrifugation at 3000 rpm at 4°C, vacuum dried, and dissolved in water. The detection limit was 10 pg/g wet weight.

Total renin (ie, renin plus prorenin) was measured by immunoradiometric assay in homogenized tissue after treatment with acid and plasmin to activate prorenin.[14]

Data Analysis

Data are expressed as mean±SEM or geometric mean and range, and n refers to the patient number. Trabeculae showing <25 mg response to norepinephrine at the start or end of the experiment were excluded from analysis. CRCs were analyzed as described previously to obtain pEC_{50} ($=-\log[EC_{50}]$) values.[13] Aldosterone levels below the detection limit were taken to be equal to the detection limit. Statistical analysis was by Mann–Whitney *U* test for unpaired observations, paired *t* test, or 1-way ANOVA, followed by post hoc evaluation according to Tukey. $P<0.05$ was considered significant.

Results

Trabeculae Studies

Baseline contractile forces were 164±11 mg and 233±15 mg in atrial (n=29) and ventricular (n=15) trabeculae, respectively. Norepinephrine increased contractile force in both types of trabeculae in a concentration-dependent manner (pEC_{50} 6.2±0.1 and 6.1±0.1, respectively). The contractile response to 10 μmol/L norepinephrine at the end of the experiment was not different from that at the start (+208±16% versus +205±11% in atrial trabeculae and +196±20% versus +198±20% in ventricular trabeculae). This indicates that tissue viability did not decrease during the course of the experiment.

Aldosterone (pEC_{50} 8.3±0.1; n=25) and hydrocortisone (pEC_{50} 7.6±0.2; n=5; $P<0.01$ versus aldosterone), but not 17β-estradiol (n=4), reduced contractility in atrial trabeculae (Figure 1). The effects of aldosterone occurred within 5 to 15 minutes after its application. Aldosterone also reduced contractility in ventricular trabeculae (pEC_{50} 8.1±0.5; n=7), although its effects in this preparation were more modest than in atrial trabeculae. The effects of spironolactone in atrial (pEC_{50} 8.2±0.4; n=7) and ventricular (pEC_{50} 8.7±0.1; n=6) trabeculae were comparable to those of aldosterone. Eplerenone did not exert effects in either atrial (n=7) or ventricular (n=4) trabeculae, nor did solvent (n=4 and 6, respectively). In atrial trabeculae, the negative inotropic effect of aldosterone after prestimulation with either forskolin (n=6) or Ang II (n=6) was identical to that at baseline. Aldosterone also relaxed forskolin-prestimulated ventricular trabeculae (n=6). Without aldosterone, the forskolin-induced increase in contractility remained stable for ≥45 minutes (ie, the time required to construct an aldosterone CRC).

Spironolactone (n=7), eplerenone (n=5), L-NAME (n=5), hydroxocobalamin (n=5), ODQ (n=4), thapsigargin (n=5), and solvent (methanol [n=4] or DMSO [n=5]) did not affect the

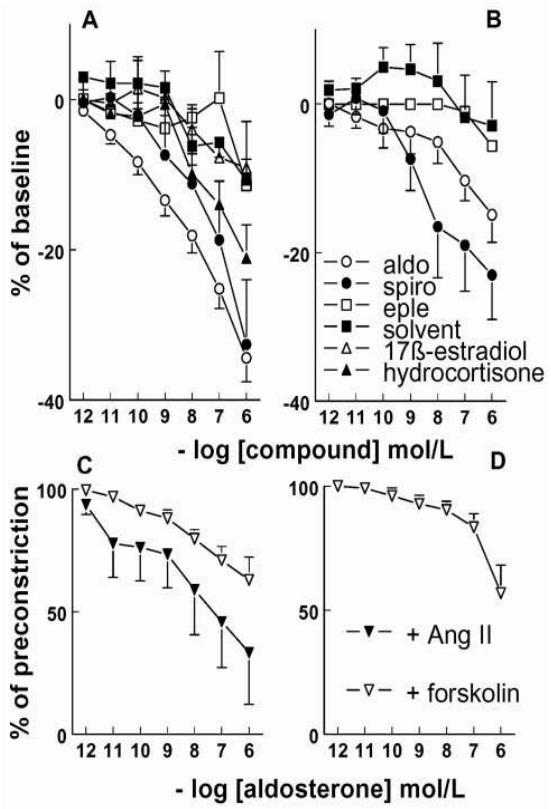

Figure 1. Inotropic effects of aldosterone (aldo), spironolactone (spiro), eplerenone (eple), solvent (ethanol), 17β-estradiol, and hydrocortisone in human right atrial (A and C) and left ventricular (B and D) trabeculae at baseline (A and B) and after prestimulation with 1 μmol/L forskolin (to 326±48% and 325±83% of baseline in atrial and ventricular trabeculae, respectively) or 100 nmol/L Ang II (to 32±7% of baseline; C and D). Data (mean±SEM of 4 to 25 experiments) are expressed as percent change from baseline contractile force or from the contractile force after prestimulation.

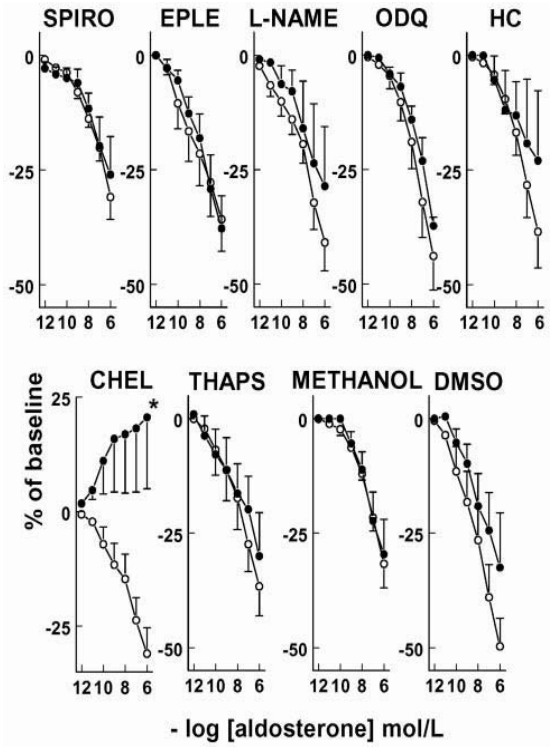

Figure 2. Inotropic effects of aldosterone in atrial trabeculae in the absence (○) or presence (●) of spironolactone (SPIRO; 10 μmol/L), eplerenone (EPLE; 1 μmol/L), L-NAME (100 μmol/L), ODQ (10 μmol/L), hydroxocobalamin (HC; 200 μmol/L), chelerythrine chloride (CHEL; 1 μmol/L), thapsigargin (THAPS; 1 μmol/L), or solvent (methanol or DMSO). Data (mean±SEM of 4 to 7 experiments) were obtained in a paired setup and have been expressed as percent change from baseline contractile force. *P<0.05 vs control.

aldosterone-induced negative inotropic effects (Figure 2), and with the exception of spironolactone (see above), none of these inhibitors affected baseline contractility. In contrast, chelerythrine chloride (n=7) not only blocked the effect of aldosterone, but tended to reverse it into a positive inotropic (P=NS) response. Chelerythrine chloride did not affect baseline contractility.

Vessel Studies

Ang II concentration-dependently constricted HCAs (pEC_{50} 7.5±0.5; maximum contractile response [E_{max}] 29±7%; n=13; Figure 3). Aldosterone (n=11), hydrocortisone (n=11), 17β-estradiol (n=10), or solvent (ethanol; n=5), at concentrations ranging from 1 pmol/L to 1 μmol/L, did not affect baseline contractility in HCAs (data not shown). However, when constructing an Ang II CRC after exposure to aldosterone or 17β-estradiol (without refreshing the organ bath fluid), E_{max} doubled to 55±11% and 51±14%, respectively (P<0.05 versus control for both), with no change in pEC_{50} (7.5±0.2 versus 7.5±0.2; Figure 3). Hydrocortisone tended to induce a similar potentiation (P=NS; Figure 3), whereas ethanol was without effect. Neither spironolactone (n=8) nor eplerenone (n=8) blocked the effect of aldosterone (Figure 3). The amount of phosphorylated ERK 1/2 tended to be increased in the presence of 1 μmol/L aldosterone, hydrocortisone, and 17β-estradiol (P=NS versus Ang II alone; n=6 for all; Figure 4), with no change in the total

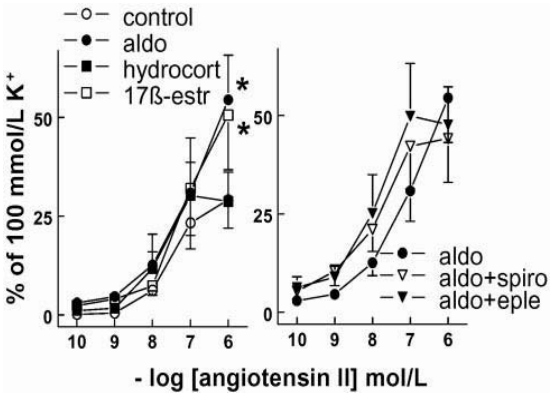

Figure 3. Contractions of HCAs to Ang II at baseline (control) or after previous exposure to aldosterone (aldo), hydrocortisone (hydrocort), or 17β-estradiol (17β-estr; 1 pmol/L-1 μmol/L) with or without preincubation with 10 μmol/L spironolactone (Spiro) or 1 μmol/L eplerenone (Eple). Data (mean±SEM; n=7 to 10) are expressed as a percentage of the response to 100 mmol/L KCl. *P<0.05 vs control.

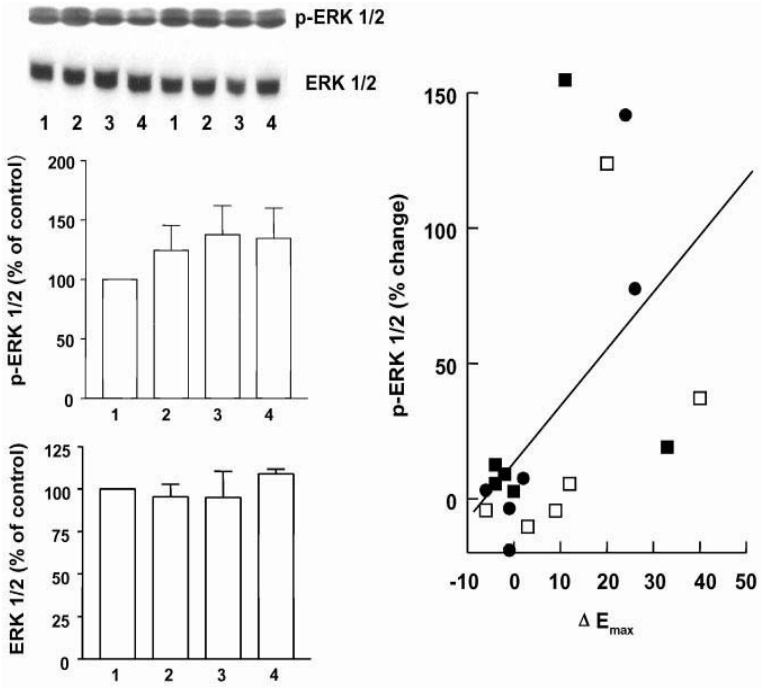

Figure 4. Left, Effects of Ang II on ERK 1/2 phosphorylation (p-ERK 1/2) and total ERK 1/2 in HCAs at baseline (1; control) or after previous exposure to 1 μmol/L aldosterone (2), hydrocortisone (3), or 17β-estradiol (4). Top, 2 representative experiments; bottom, mean±SEM of 6 experiments. Right, Correlation (r=0.56; P<0.01) between the change in E_{max} (Δ E_{max}) and the percent change in Ang II–induced ERK 1/2 phosphorylation in HCAs after previous exposure to aldosterone (●), hydrocortisone (■), or 17β-estradiol (□).

amount of ERK 1/2. The increase in ERK 1/2 phosphorylation was limited to vessel segments displaying a large increase in E_{max}, as evidenced by the significant correlation between ΔE_{max} and ERK 1/2 phosphorylation (r=0.56; P<0.01; Figure 4).

The effects of aldosterone, 17β-estradiol, and hydrocortisone toward Ang II could be reproduced in PRAs: none of these steroids affected baseline contractility at concentrations ranging from 1 pmol/L to 1 μmol/L, and only previous exposure to aldosterone and 17β-estradiol increased the Ang II E_{max} (n=9; Figure 5). Therefore, PRAs were used to test whether the effects of aldosterone and 17β-estradiol could also be observed at lower concentrations. Preincubation with 17β-estradiol concentrations <1 μmol/L did not result in potentiation (n=7; data not shown). In contrast, the effects of aldosterone could be mimicked at concentrations as low as 1 nmol/L (n=10; P<0.05) or, in the presence of L-NAME, 1 pmol/L (n=4; P<0.05; Figure 5). Furthermore, the potentiating effects were specific for Ang II because preincubation with 1 nmol/L aldosterone did not affect the phenylephrine CRC (n=4; Figure 5).

Aldosterone Levels in the Human Heart

Aldosterone levels in failing hearts (184 [range 10 to 4710] pg/g; n=11) were ≈10-fold higher (P<0.02) than in normal hearts (26 [range 10 to 481] pg/g; n=12). This parallels our previous observations on cardiac renin.[14] In fact, the levels of renin in the heart correlated significantly with those of aldosterone (r=0.71; P<0.05; Figure 6).

Discussion

The present study is the first to demonstrate nongenomic actions of aldosterone in the human heart. Aldosterone exerted a negative inotropic effect in atrial and ventricular trabeculae at baseline and after prestimulation with the adenylyl cyclase activator forskolin. This effect was long lasting and, unlike the short-lasting negative inotropic effect of bradykinin,[15] did not involve the NO–cGMP pathway. The PKC inhibitor chelerythrine chloride fully blocked the aldosterone-induced negative inotropy, suggesting that it is mediated via the diacylglycerol–PKC signal transduction pathway. Patch clamp studies in rabbit ventricular myocytes have revealed that aldosterone affects Na^+-K^+ pump activity via ϵPKC,[12] thereby providing a mechanism for the negative inotropic response to aldosterone. The modest effect of aldosterone in ventricles is in agreement with the fact that atria have fewer sodium pumps than ventricles[16] because low expression enhances inotropic sensitivity.

The lack of effect of MR antagonists suggests that the inotropic effects of aldosterone occur in an MR-independent manner. In fact, as in the isolated perfused rat heart,[4,5] spironolactone exerted an inotropic effect that was comparable to that of aldosterone, whereas the more selective MR antagonist eplerenone did not. This further supports the MR independency of the effects of aldosterone and spironolactone. However, importantly, aldosterone and spironolactone exerted positive inotropy in the rat heart,[4,5] as opposed to the negative inotropy observed here and in rabbit cardiomyocytes.[12] Although this may relate to species differences, alternative explanations must be considered. First, inotropic effects in isolated trabeculae do not necessarily parallel inotropic effects in intact hearts because the latter also reflect responses on coronary flow. Second, similar diametrically differing effects of aldosterone have been observed on flow, either because such effects involve different cells or because different second messengers are activated depending on the experimental circumstances.[5,6,12,17] Finally, the consequences of PKC-induced regulation of Na^+-K^+ pump activity are tissue specific and range from stimulation to inhibition or no change.[12,18]

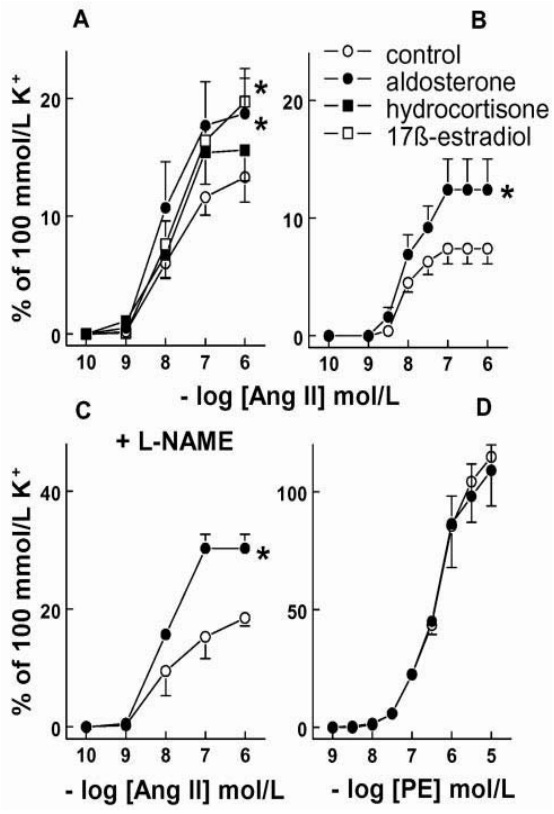

Figure 5. A, Contractions of PRAs to Ang II at baseline (control) or after previous exposure to aldosterone, hydrocortisone, or 17β-estradiol (1 pmol/L to 1 μmol/L). B, Contractions of PRAs to Ang II at baseline (control) or after preincubation with 1 nmol/L aldosterone. C, Contractions of PRAs to Ang II at baseline (control) or after preincubation with 1 pmol/L aldosterone and 100 μmol/L L-NAME. D, Contractions of PRAs to phenylephrine (PE) at baseline (control) or after preincubation with 1 nmol/L aldosterone. Data (mean±SEM; n=4 to 10) are expressed as a percentage of the response to 100 mmol/L KCl. *P<0.05 vs control.

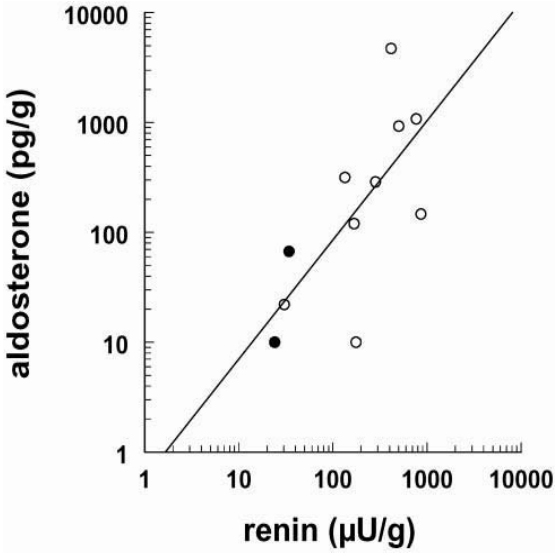

Figure 6. Correlation between the left ventricular levels of renin and aldosterone (r=0.71; P<0.05) in 9 failing (○) and 2 (●) normal human hearts.

Hydrocortisone mimicked the inotropic effects of aldosterone at lower potency. This underlines the specificity of the aldosterone effect. However, because glucocorticoids circulate at levels that are several orders of magnitude higher than those of aldosterone, the inotropic effects of aldosterone and glucocorticoids may occur simultaneously in vivo.

The aldosterone levels in failing human hearts were found to be up to ≈5000 pg/g (corresponding with ≈15 nmol/L), that is, high enough to allow the inotropic effects of aldosterone to occur under pathological conditions in vivo. The levels in normal hearts (≈25 pg/g or <0.1 nmol/L) appeared to be too low to exert inotropic effects. Although the origin of aldosterone in the heart is still under debate,[8] our observation that the cardiac aldosterone levels correlate with the cardiac levels of renin (which is exclusively of renal origin[14]), combined with previous findings on cardiac extraction of aldosterone,[19] suggest that at least some cardiac aldosterone is of extracardiac origin.

The high renin levels in failing human hearts will result in high local Ang II levels.[20] In agreement with previous studies,[21] Ang II was found to induce a modest (compared with norepinephrine) positive inotropic effect. Aldosterone counteracted this effect. This observation contrasts with the aldosterone-induced potentiation of the vasoconstrictor effect of Ang II in HCAs. Hydrocortisone and 17β-estradiol similarly potentiated constriction, although significance was reached for 17β-estradiol only. The magnitude of potentiation (ie, the increase in E_{max}) correlated with the increase in the level of phosphorylated ERK 1/2, in full agreement with a previous study on steroid–Ang II interaction in rat aortic vascular smooth muscle cells.[7] None of the steroids applied in the present study exerted a constrictor or dilator effect of its own, and the Ang II–potentiating effects became apparent only after constructing a CRC to the steroid. Such construction requires 30 to 60 minutes, a time interval that is insufficient to allow the AT_1 receptor upregulation that underlies the Ang II potentiation after a 24- to 48-hour exposure to aldosterone and glucocorticoids.[11,22]

Studies in HCA smooth muscle cells have already indicated that Ang II is capable of activating MR-mediated gene expression in an aldosterone-independent manner (suggesting MR activation by post-translational modifications such as phosphorylation).[10] The present data extend this observation by demonstrating functional synergy when applying aldosterone together with Ang II. This synergy did not depend on MR activation, was selective, and occurred at physiological aldosterone levels.

In the rat aorta,[3] as well as the human forearm,[6] aldosterone has been reported to enhance vasoconstriction and vasodilation. The latter was endothelium dependent, whereas the former could only be observed during NO synthase blockade. The absence of aldosterone-induced vasodilation in HCAs may relate to our inability to observe endothelial NO release in these vessels.[23]

Limited NO release could also underlie the absence of a vasodilator response to 17β-estradiol in HCAs. Despite the higher incidence of stroke, myocardial infarction, and dementia in postmenopausal women taking hormone replacement therapy,[24] virtually all in vitro studies published so far claim that estrogen induces vasodilation through endothelium-dependent or endothelium-independent mechanisms.[24] An alternative ex-

planation for the absence of estrogen-induced vasodilation in HCAs, at least in women, is the change in estrogen receptor cellular localization during perimenopause.[25] Furthermore, estrogen inactivates reactive oxygen species like the vasodilator H_2O_2[26,27] and alters the AT_1/AT_2 receptor ratio.[28,29] Both phenomena will directly affect Ang II–induced vasoconstriction.

Perspectives

Aldosterone induces a negative inotropic response in human trabeculae (thereby antagonizing the positive inotropic actions of Ang II) and potentiates the vasoconstrictor effect of Ang II in HCAs. These effects occur in a rapid, nongenomic manner, independently of MR, and involve PKC and ERK 1/2, respectively. Combined with the nonhemodynamic effects of aldosterone in cardiac tissue, which result in inflammation and fibrosis,[5,30] these data shed light on the wide variety of actions of aldosterone in the heart. Future studies should now address which receptor mediates the nongenomic cardiac effects of aldosterone and to what degree these effects occur in failing hearts. In view of the comparable beneficial effects of spironolactone and eplerenone in heart failure,[1,2] such studies should also critically evaluate the physiological importance of the negative inotropic effect of spironolactone. The many nongenomic, non–MR-mediated effects of aldosterone suggest that aldosterone synthase inhibitors may yield effects on top of MR blockade.

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Chapter 7

Aldosterone synthase gene (*CYP11B2*) C-344T polymorphism and cardiac hypertrophy in subjects with hypertrophic cardiomyopathy

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Submitted

Summary

The development of left ventricular (LV) hypertrophy in subjects with hypertrophic cardiomyopathy (HCM) is variable, suggesting a role for modifying factors such as angiotensin (Ang) II. Indeed, both the Ang II type 1 receptor (AT_1-R) A/C^{1166} polymorphism and the Ang II type 2 receptor (AT_2-R) A/C^{3123} polymorphism modulate phenotypic expression of hypertrophy in HCM. Recently, aldosterone has been implicated as a major link between sarcomeric mutations and cardiac phenotype. To determine whether aldosterone modulates hypertrophy in HCM, aldosterone was measured in left ventricular (LV) tissue of 12 organ donors and 8 patients with HCM. The effect of the aldosterone synthase gene (*CYP11B2*) C-344T polymorphism on LV mass index (LVMI) and interventricular septum thickness (IVS) was determined in 79 genetically independent subjects with HCM. LVMI and IVS were measured by 2-D echocardiography. Extent of hypertrophy was quantified by a point score (Wigle score). Prorenin, renin, and ACE were determined by immunoradiometric or fluorimetric assays, and genotyping was performed by PCR. Aldosterone in HCM hearts equalled aldosterone in normal hearts hearts. In women with HCM, no associations between *CYP11B2* genotype and any of the measured parameters were observed, whereas in men with HCM, LVMI increased with the presence of the T allele (LVMI=137±9 and 178±9 g/m^2 in men with the CC vs. the CT+TT genotype, respectively; P=0.06). Similar T allele-related increases in men were observed for IVS (P=0.01) and Wigle score (P=0.08), but not for prorenin, renin or ACE. Multiple regression analysis revealed that the aldosterone synthase gene T allele-related effect on IVS occurred independently (P=0.04) of renin, ACE, the AT_1-R gene A/C^{1166} polymorphism and the AT_2-R A/C^{3123} polymorphism. We conclude that aldosterone modulates cardiac hypertrophy in men with HCM, thereby raising the need for studies determining the benefit of mineralocorticoid receptor blockade in HCM.

Introduction

Hypertrophic cardiomyopathy (HCM) is a genetic disease characterized by unexplained cardiac and myocyte hypertrophy, interstitial fibrosis and myocyte disarray [1]. Mutations in at least 11 different sarcomeric proteins have been identified as the primary defect [2]. Yet, even if patients have identical causative genotypes, they still vary considerably by phenotype [3]. Other factors, genetic as well as environmental, may therefore modify the phenotypic expression of the mutated gene. Angiotensin (Ang) II, the end-product of the renin-angiotensin system (RAS), is among these factors. It modulates cardiac hypertrophy in HCM both via growth-stimulatory Ang II type 1 (AT_1) receptors and via growth-inhibitory [4] Ang II type 2 (AT_2) receptors [5,6]. Its effects are gender-specific, and occur independently of the circulating RAS.

A recent study suggests that aldosterone, similar to Ang II, acts as a major link between sarcomeric mutations and cardiac phenotype in HCM [7]. According to this study, the myocardial aldosterone levels in humans with HCM are 4-fold elevated, and aldosterone provokes expression of hypertrophic markers in rat cardiac myocytes and of collagens in rat cardiac fibroblasts. The latter effects occurred in a mineralocorticoid receptor (MR)-dependent manner, as they could be blocked by the MR antagonist spironolactone [7,8]. Aldosterone, like Ang II [9], may be produced locally in the heart [10], and the aldosterone synthase (*CYP11B2*) polymorphism associates with left ventricular mass (LVM) in human essential hypertension [11]. Furthermore, MR do occur in the human heart [12].

It was the aim of the present study to further determine the role of aldosterone as a modulator of hypertrophy in HCM. We first measured aldosterone in cardiac tissue obtained during surgery of patients with HCM. Secondly, we determined the relationship between the aldosterone synthase (*CYP11B2*) polymorphism and cardiac hypertrophy in 79 HCM patients.

Methods

Patients

All studies were approved by the internal review board and patients gave informed consent. One-hundred and seventeen patients with HCM (age 21-81 years) visiting the HCM Clinic at the Erasmus MC between 1994 and 1997 for a routine follow-up were included. HCM had been diagnosed on the basis of echocardiographic criteria showing a nondilated, hypertrophied left ventricle (any wall thickness >15 mm) in the absence of known causes of left ventricular hypertrophy [13]. DNA quality and quantity allowed genotyping in 81 patients. Of these patients, 31 had a sporadic form of HCM and 36 had at least one other affected first degree family member. The family history of HCM was unknown in 14 patients. To avoid potential bias introduced by the presence of genetically dependent samples (relatives), we randomly selected one patient per family. This resulted in a final cohort of 79 genetically independent patients, of whom 5 were receiving an ACE inhibitor, 22 a β-adrenergic antagonist, 39 a calcium-channel blocker and 9 a diuretic.

Left ventricular tissue was obtained from 8 HCM subjects (3 men, 5 women, age 36±3 (range 16-57) years) undergoing septal myectomy [14] and from 12 heart-beating organ donors (5 men, 7 women, age 39±3 (range 17-54) years), who died of non-cardiac causes (9 cerebrovascular accident, 1 head trauma, 2 brain hypoxia) <24 hours before the heart was taken to the laboratory. Tissue pieces (1-10 g) were kept at -70°C.

Echocardiographic Methods

Two-dimensional echocardiography was performed with commercially available equipment (Toshiba Sonolayer). Images were recorded on videotape for off-line analysis by 2 physicians who were blinded to the genotyping results. Interventricular septal thickness (IVS) and LVM were determined as described before [5]. LVM was indexed (LVMI) to body surface area (BSA). Peak left ventricular outflow tract gradient at rest was estimated using the modified Bernoulli equation [5]. Since echocardiographic measurement of LVMI may not truly reflect the extent of hypertrophy and the involvement (or lack thereof) of the distal (apical) half of the septum or lateral wall, the

extent of hypertrophy was also assessed by a semi-quantitative point score (range 0-10) method developed by Wigle et al. [15].

Biochemical Measurements

Prorenin and renin were quantified in peripheral venous blood using an immunoradiometric assay kit (Nichols Institute) [16]. Prorenin and renin are expressed as mU/L, using the human kidney renin standard MRC 68/356 as a reference. ACE activity was measured with a commercial kit (ACE Color) [17]. Aldosterone was measured by solid-phase radioimmunoassay (DPC) following its extraction from left ventricular tissue. In short, tissue was homogenized 1:2 in methanol. The supernatant was collected after a 15-min centrifugation at 3,000 rpm at 4ºC, vacuum dried, and dissolved in water. The detection limit was 10 pg/g wet weight, and levels below the detection limit were taken to be equal to the detection limit.

Genetic Analysis

Peripheral leukocytes were used to isolate genomic DNA in H_2O using the QIAamp Bloodkit (QIAGEN Inc.). The aldosterone synthase gene (*CYP11B2*) C-344T polymorphism was determined according to Barbato et al.[18]. Polymerase chain reaction (PCR) amplifications were carried out in a 50 µl reaction volume, using 10 ng of genomic DNA. Each reaction contained 1x PCR buffer II (Perkin Elmer), 1.5 mmol/l $MgCl_2$, 0.2 mmol/l each of the deoxynucleotide triphosphates (Roche), 1.25 U of Amplitaq Gold (Perkin Elmer) and 40 pmol each of forward primer 5'-CAGGAGGAGACCCCATGTGAC-3' and reverse primer 5'-CCTCCACCCTGTTCAGCCC-3'. PCR consisted of initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 67°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension at 72°C for 5 min. For restriction fragment length polymorphism (RFLP) determination, the PCR product (10 µl) was digested with *Hae III* for 2 hours at 37°C. The fragment sizes were analyzed on a 4% agarose gel with ethidium bromide staining. The PCR-RFLP was validated by direct sequencing of PCR product from a heterozygote sample, using the Big Dye Terminator

Cycle Sequencing Kit (Applied Biosystems) on a ABI 310 capillary sequencer (Applied Biosystems). The ACE I/D polymorphism, the AT_1 receptor A/C^{1166} polymorphism, and the AT_2 receptor A/C^{3123} polymorphism were determined as described before [5,6].

Statistical Analysis

Data are expressed as mean±SEM or geometric mean and range. Analysis was performed with the SPSS 11.0 statistical package. Hardy-Weinberg equilibrium was tested by X^2 test. Univariate and multiple regression analyses were conducted to determine the percentage of explained variance in LVMI and IVS that is accounted for by the genotypes of the candidate modifier genes and other variables. In the multiple regression analysis the RAS gene polymorphisms, age, peak left ventricular outflow tract gradient and renin concentration were tested as independent variables. Prorenin and ACE were excluded from this analysis because of their high correlations with renin (r=0.68, $P<0.001$) and ACE genotype (r=0.39, $P=0.003$), respectively.

Results

Cardiac aldosterone levels in HCM patients (27 (10-233) pg/g, n=8) were not different from the aldosterone levels in hearts of organ donors (26 (10-481) pg/g, n=12).

Table 1 lists the characteristics of the HCM patients by *CYP11B2* genotype. Frequencies of the C and T allele (0.41 and 0.59, respectively) were similar to previously reported numbers in normal white populations [11,18,19], and genotype frequencies were in agreement with Hardy-Weinberg equilibrium. The percentage of patients taking ACE inhibitors, β-adrenergic antagonists, calcium channel blockers or diuretics did not differ between the various groups (data not shown). When analyzing all subjects together, no genotype-related differences were observed with regard to any of the measured parameters (Table 1), in full agreement with a previous study [20]. However, subdivision of the population according to gender revealed that IVS was significantly higher ($P=0.01$)

Table 1 *Characteristics of HCM patients according to aldosterone synthase genotype*.

Parameter	Genotype		
	CC (n=10)	CT (n=45)	TT (n=24)
Sex, M/F	7/3	24/21	14/10
Age, y	48±5	51±2	48±4
BSA, m^2	1.88±0.05	1.81±0.03	1.88±0.04
IVS, mm	19.9±1.9	21.5±0.6	22.1±1.0
LVMI, g/m^2	152±16	178±8	169±10
Wigle score, 1-10	5.8±0.7	6.5±0.3	6.2±0.5
Gradient, mm Hg	33.6±8.6	54.8±6.0	53.0±8.3
Prorenin, mU/L	289±119	198±19	181±15
Renin, mU/L	43.1±18.0	23.4±2.0	22.3±2.2
ACE, U/L	11.7±1.0	9.8±0.3	10.0±0.5

BSA, body surface area; IVS, interventricular septum thickness; LVMI, left ventricular mass index; gradient, peak left ventricular outflow tract gradient.

Table 2 *Characteristics of male and female HCM patients according to aldosterone synthase genotype.*

Parameter	**Genotype**			
	Men		Women	
	CC	CT+TT	CC	CT+TT
n	7	38	3	31
Age, y	46±6	47±2	51±3	54±3
BSA, m^2	1.88±0.05	1.81±0.03	1.88±0.04	1.88±0.04
IVS, mm	17.6±1.0	21.9±0.7*	25.3±5.8	21.5±0.9
LVMI, g/m^2	137±9	178±9¶	183±53	172±9
Wigle score, 1-10	5.0±0.8	6.4±0.3º	7.7±1.5	6.3±0.5
Gradient, mm Hg	33.3±12.3	51.0±6.5	34.3±13.2	57.8±7.8
Prorenin, mU/L	346±167	206±20	91±4	173±16
Renin, mU/L	51.3±25.4	24.7±1.9	14.4±1.0	20.5±2.4
ACE, U/L	14.7±2.0	10.1±0.4	8.6±0.0	9.6±0.3

*BSA, body surface area; IVS, interventricular septum thickness; LVMI, left ventricular mass index; gradient, peak left ventricular outflow tract gradient. *P=0.01, ¶P=0.06, ºP=0.08 vs. CC men.*

in men carrying 1 or 2 T alleles than in male CC homozygotes. Similar trends were observed for LVMI (P=0.06) and Wigle score (P=0.08). No such T allele-related effects were observed in women, nor did any of the other parameters correlate with the presence of the T allele in either men or women. Univariate regression analysis showed that *CYP11B2* genotype accounted for 16.3% of the variablity of IVS (r=0.40, P<0.01). Multiple regression analysis revealed that this effect occurred independently (β=3.7±1.7; P=0.04) of renin, the ACE gene polymorphism, the AT_1-R gene A/C^{1166} polymorphism, the AT_2-R A/C^{3123} polymorphism, age and peak left ventricular outflow tract gradient.

Discussion

The present study shows that aldosterone, like Ang II, is among the factors that modify the phenotypic expression of the mutated gene in HCM. IVS was higher in male HCM subjects carrying the *CYP11B2* T allele, and similar observations were made for LVMI and Wigle score. This effect occurred independently of the RAS. Contrary to our expectation, and opposing the recent study by Tsybouleva et al. on this subject [7], the left ventricular tissue levels of aldosterone in subjects with HCM were not significantly different from those in age-matched controls.

Our cardiac aldosterone levels resemble the levels reported by Gomez-Sanchez et al. [21] and Fiebeler et al. [22] in the rat heart. These authors observed a close relationship between the levels of aldosterone in the heart and in blood plasma, and they concluded that, at least in the rat, cardiac aldosterone is largely, if not completely, of adrenal origin. The plasma levels of aldosterone in subjects with HCM, like those of renin, are in the normal range (≈30-200 pg/ml) [7,23,24], and thus no alteration in the cardiac aldosterone content of HCM subjects would be expected if cardiac aldosterone were exclusively of adrenal origin. Based on *CYP11B2* mRNA measurements however, Tsybouleva et al. [7] have suggested that cardiac aldosterone is of local origin. In contrast with this conclusion, their cardiac aldosterone levels were <0.1 pg/g protein, i.e. >2 orders orders of magnitude below the levels that are minimally expected based on the presence of blood in cardiac tissue [25,26]. Thus, conclusive evidence for cardiac production of aldosterone in subjects with HCM is still lacking.

The association between *CYP11B2* genotype and cardiac hypertrophy in HCM parallels the association between the *CYP11B2* T allele and LVM in subjects with essential hypertension [11]. It also extends a previous study on this polymorphism in HCM subjects which showed no significant T allele-related increase in LVMI [20]. The latter study did not evaluate the gender-specificity of the effect. In addition, since the influence of the *CYP11B2* genotype, like that of the ACE I/D genotype, will depend on the gene mutation that determines the primary defect [27], *CYP11B2* T allele-related effects do not necessarily apply to all HCM populations.

According to most [11,18,19,28,29] (but not all [19,30]) studies, the *CYP11B2* T allele is associated with elevated plasma aldosterone levels, while serum aldosterone associates with the variability of LVM in both healthy controls and subjects with hypertension [31]. Thus, a picture arises in which the T allele results in elevated plasma levels of aldosterone, which subsequently affect cardiac hypertrophy. Indeed, there is ample evidence suggesting that circulating aldosterone acts as a pro-inflammatory, hypertrophic and profibrotic factor in the heart [7,8,32,33]. In further support of this concept, MR occur in the human heart [12] and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) activity associates directly with LVM in essential hypertension [34]. 11β-HSD2 inactivates cortisol and corticosterone, thereby preventing these glucocorticoids from stimulating MR. The 11β-HSD2 levels are relatively low in the heart [12], whereas the glucocorticoid concentrations in blood are several orders of magnitude above those of aldosterone. Thus, activation of cardiac MR by circulating aldosterone is possible only in the presence of sufficiently high 11β-HSD2 activity. Importantly, the above scenario does not require the cardiac aldosterone levels to be elevated in HCM subjects as compared to controls, since the genotype distribution in our HCM population was similar to that in normal white populations [11,18,19].

The effect of the T allele on cardiac hypertrophy occurred independently of circulating renin, ACE and the two AT receptors, and thus aldosterone exerts additive effects on top of Ang II, despite earlier studies suggesting that aldosterone exerts its effects via Ang II or AT_1 receptors and vice versa [35-37]. This conclusion is in full agreement with the renin-independent associations between LVM, *CYP11B2* genotype and 11β-HSD2 activity in hypertension [11,34]. The gender-specificity of the association

in our study is more difficult to explain. Estrogen may mimic or antagonize some of the effects of aldosterone [38], whereas the well-known gender-related differences in renin and angiotensinogen [24,39] will affect, through Ang II, the biosynthesis of aldosterone.

Finally, the MR antagonist spironolactone has been demonstrated to reduce the extent of myocyte disarray and to reverse interstitial fibrosis in a transgenic mouse model of human HCM mutation (cTnT-Q92 mice) [7]. Interestingly, these mice did not display elevated cardiac aldosterone levels [7]. Combined with the results from the present study, these data raise the need for studies determining the benefit of MR blockade in HCM.

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Chapter 8

Summary, General Discussion and Future Studies

Summary

Introduction and aim (Chapter 1)

Classically, aldosterone is an adrenal-derived steroid that acts through intracellular mineralocorticoid receptors (MR) in the kidney. Angiotensin (Ang) II, synthesized locally in the adrenal or derived from the circulation, is among the factors that stimulate aldosterone synthesis. Two recent clinical trials with MR antagonists have shown beneficial effects of these drugs on top of renin-angiotensin system blockade, which cannot be attributed solely to blockade of the renal MR-mediated effects on blood pressure. Therefore, it is now generally assumed that aldosterone also exerts actions outside the kidney.

This chapter summarizes the recent findings in this area, focusing also on the idea that aldosterone exerts extrarenal actions through a novel (so far unidentified) membrane receptor. A distinction is made between the classic 'genomic' (occurring after hours and involving DNA-directed, RNA-mediated protein synthesis) and the novel 'non-genomic' (occurring with minutes and not involving DNA-directed, RNA-mediated protein synthesis) effects of aldosterone. Another controversial issue is whether aldosterone synthesis also occurs outside the adrenal, e.g. in the heart. Aldosterone synthase expression has been demonstrated in various non-adrenal cells.

It was the aim of the present study to unravel first to what degree locally or blood-derived Ang II contributes to adrenal aldosterone synthesis, and whether this local production of Ang II, if occurring, takes place intra- or extracellularly. Second, we studied the genomic and non-genomic effects of aldosterone in the rat heart, under normal and ischemic conditions, and we verified the source of cardiac aldosterone. Third, we investigated the effects of aldosterone in human myocardial trabeculae and coronary arteries and the second messengers mediating these effects. We also compared the cardiac levels of aldosterone in healthy and diseased human hearts, and we investigated the association of the aldosterone synthase gene (CYP11B2) C-344T polymorphism with cardiac hypertrophy in subjects with hypertrophic cardiomyopathy.

Origin and site of synthesis of Ang II in the adrenal (Chapters 2 and 3)

Infusions of ^{125}I-radiolabeled angiotensins in pigs revealed that circulating ^{125}I-Ang II, but not circulating ^{125}I-Ang I, accumulates in the adrenal, reaching steady-state levels that are 15-20 times higher than its levels in blood plasma. Yet, the levels of endogenous Ang II are almost 400 times higher than the Ang II levels in blood, and, thus, the majority of adrenal Ang II is synthesized locally from locally generated Ang I. The Ang II type 1 (AT_1) receptor antagonist eprosartan greatly reduced the adrenal uptake of circulating ^{125}I-Ang II, and increased plasma Ang II to a greater degree than tissue Ang II. As a consequence, eprosartan equally reduced the tissue/plasma concentration ratios of both Ang II and ^{125}I-Ang II.

From these data it can be concluded that adrenal Ang II generation occurs extracellularly, and is followed by internalization via AT_1 receptor-mediated endocytosis. Apparently therefore, the truncated prorenin (with full enzymatic activity) that occurs intracellularly in adrenal tissue, exerts functions other than generating Ang I, in agreement with the fact that angiotensinogen-synthesizing cells release angiotensinogen into the extracellular space rather storing it intracellularly.

Effects of aldosterone in the rat heart (Chapters 4 and 5)

In the isolated perfused rat Langendorff heart, aldosterone, like Ang II, rapidly increased left ventricular pressure and decreased coronary flow. The MR antagonists spironolactone and eplerenone did not block these effects. In fact, spironolactone exerted similar inotropic effects on top of aldosterone, and thus it appears that the effects of aldosterone on inotropy and flow occur in a 'non-genomic' (non-MR-mediated) manner.

Both spironolactone and eplerenone greatly improved the condition of the heart following ischemia and reperfusion (infarct size ↓, left ventricular pressure recovery ↑, arrhythmia incidence ↓), suggesting that these drugs interfere with the deleterious effects of endogenous aldosterone. Perfusion studies with aldosterone showed that the steroid rapidly accumulated in cardiac tissue, not only in extracellular (interstitial) fluid, but also in a second, as yet unidentified, compartment. Washout from this second compartment occurred relatively rapid (half life < 10 minutes), and after prolonged washout the cardiac

aldosterone levels were close to or below the detection limit. Thus, the majority of cardiac aldosterone is blood-derived.

In cultured aortic smooth muscle cells, low concentrations of aldosterone enhanced the effect of Ang II on DNA synthesis. Higher aldosterone concentrations reduced DNA synthesis, both in smooth muscle cells and in cardiac myocytes. High aldosterone concentrations also reduced collagen synthesis in cardiac fibroblasts. Spironolactone blocked the effects on both DNA and collagen synthesis, suggesting that they are MR-mediated and of 'genomic' nature.

In conclusion, aldosterone induces positive inotropic and vasoconstrictor effects in the rat heart in a non-genomic manner, and these effects are comparable to those of Ang II. MR stimulation reduces DNA and collagen synthesis in cardiac cells, and worsens the condition of the heart post-coronary artery occlusion. Interference with the latter phenomena may underlie, at least in part, the beneficial actions of MR antagonists in heart failure.

Effects of aldosterone in the human heart (Chapters 6 and 7)

In human myocardial trabeculae, aldosterone induced a negative inotropic response, in apparent contrast with its positive inotropic effects in the rat heart. The protein kinase C (PKC) inhibitor chelerythrine chloride, but not the MR antagonists spironolactone or eplerenone, blocked this negative inotropic effect, suggesting that it is mediated via a non-MR in a PKC-dependent manner. The aldosterone concentrations required to induce this effect were in the high nanomolar range, i.e. a range that occurred in failing hearts only.

The cardiac aldosterone levels correlated with the cardiac levels of renin (which is exclusively of renal origin). Possibly therefore, cardiac aldosterone, like cardiac renin, is of extracardiac origin. In support of this concept, the cardiac and plasma concentrations of aldosterone in subjects with hypertrophic cardiomyopathy were identical to those in normal subjects, despite the fact that the aldosterone synthase gene (CYP11B2) C-344T polymorphism associated with cardiac hypertrophy in these subjects. The most likely explanation of this finding is that the T allele-related increases in plasma aldosterone are

the underlying cause of the relationship between the T allele and cardiac hypertrophy, presumably because they lead to increased cardiac aldosterone levels.

In human coronary arteries, aldosterone exerted no constrictor or dilator effect by itself. However, prior exposure to 1 μmol/L aldosterone greatly enhanced the constrictor response to Ang II. At the second messenger level, this was reflected by an increase in the level of phosphorylated ERK 1/2. Hydrocortisone and 17β-estradiol induced similar potentiating effects, but only in the case of aldosterone these effects occurred at the subnanomolar level, i.e., in a physiological range.

In summary, aldosterone induces a negative inotropic response in human trabeculae (thereby antagonizing the well-known positive inotropic actions of Ang II) and potentiates the vasoconstrictor effect of Ang II in coronary arteries. These effects occur in a non-genomic manner and involve PKC and ERK 1/2, respectively.

General Discussion and Future Studies

Truncated prorenin

Although virtually all adrenal Ang II is located intracellularly, our data do not support the concept of intracellular angiotensin generation. Thus, it remains unclear why truncated prorenin is transported into mitochondria (the site of aldosterone biosynthesis) in the adrenal [1]. Truncated prorenin has been demonstrated elsewhere in the body [2], and it is not known to what degree it occurs in circulating blood. Interestingly, truncated prorenin is enzymatically active, because it has a prosegment of insufficient length, which does not allow full coverage of the enzymatic cleft. Future studies should now investigate whether truncated prorenin is released into the extracellular space, and, if so, whether this 'prorenin' contributes to extracellular angiotensin generation. This concept is even more important now that a (pro)renin receptor has been discovered [3]. This receptor allows prorenin to display full enzymatic activity once it is bound to the receptor. Antibodies directed against various regions of the prosegment are required to address this issue [4].

Cardiac inotropy

Although aldosterone exerts opposite inotropic effects in the human and rat heart, a common second messenger pathway (the PKC – inositol 1,4,5-trisphosphate (IP_3)-1,2 diacylglycerol (DAG) pathway) may underlie both phenomena. This pathway leads to an increase in intracellular Ca^{2+} and stimulation of the Na^+/H^+ exchanger [5-10]. The latter stimulation causes a rise in intracellular Na^+. This subsequently activates Na^+/K^+-ATPase, thereby inducing a negative inotropic effect. During blockade of the rise in intracellular Na^+ however, aldosterone decreases Na^+/K^+-ATPase activity in a PKC-dependent manner [9], thus inducing a positive inotropic effect. Future studies, making use of inhibitors of the rise in intracellular Na^+ and/or Na^+/K^+-ATPase, should now address this issue in further detail. Such studies should also evaluate the PKC subtype and the interaction (synergy or antagonism) with Ang II, in particular because Ang II appears to activate the same intracellular signaling pathway [11].

Furthermore, although both spironolactone and eplerenone did not block the inotropic effects of aldosterone, it has been reported that a closed ring MR antagonist such as spironolactone is ineffective in acute in vitro experiments, whereas an open ring, water-soluble MR antagonist is effective under such conditions [5]. Thus, more work with other MR antagonists is needed to fully exclude a role for the MR in the inotropic response of aldosterone.

Coronary vasoconstriction; interaction with angiotensin II

The aldosterone-induced potentiation of Ang II, both in human coronary arteries and rat vascular smooth muscle cells, needs to be explored in further detail, in vitro as well as in vivo. Our studies in porcine renal arteries have already demonstrated that the potentiating effects of aldosterone occur at picomolar concentrations. Future investigations should now address to what degree this potentiation concerns aldosterone-induced endothelial dysfunction [12,13], and/or an interaction at the level of smooth muscle cells, involving some or all of the mediators that have recently been coupled to aldosterone, e.g., the PKC – IP_3 – DAG pathway, Na^+/H^+ exchange, Na^+/K^+-ATPase, p38 MAP kinase, reactive oxygen species and/or the epidermal growth factor receptor [7,14,15]. More work with

other MR antagonists is needed to fully exclude a role for the MR in this potentiation, as described above. Venous occlusion plethysmography studies using the forearm might substantiate the Ang II-aldosterone interaction in vivo, e.g., during treatment with MR antagonists and/or AT_1 receptor antagonists. Finally, the possibility of aldosterone-induced, NO-mediated vasodilation, proposed by several investigators [7,16], needs to be addressed more extensively, preferably making use of vessels that display excellent endothelial function [17].

Clinical relevance

The protective effect of MR antagonism in the Langendorff heart during ischemia and reperfusion most likely relates to the suppression of arrhythmias, in full agreement with the recent observation that conditional MR overexpression in the mouse heart results in life-threatening arrhythmias [18]. It cannot be explained on the basis of the vasoconstrictor effect of aldosterone, as proposed by Fujita et al. [19], unless our inability to block aldosterone-induced vasoconstriction in vitro with either spironolactone or eplerenone does not apply in vivo. A suppression of arrhythmias through MR antagonism would offer an explanation for the reduction in sudden death in RALES and EPHESUS [20,21].

In view of the comparable outcome of RALES and EPHESUS, future studies should also critically evaluate the physiological importance of the inotropic effects of spironolactone. If indeed the non-genomic effects of aldosterone on inotropy and vasoconstriction are mediated through a non-MR, aldosterone synthase inhibitors might yield effects on top of MR blockade. Obviously, the identity of the non-MR, if existing, should be established as soon as possible.

Finally, the association between the aldosterone synthase (CYP11B2) T allele and cardiac hypertrophy in subjects with hypertrophic cardiomyopathy raises the need for studies determining the benefit of MR blockade in this disease. In favor of this concept, MR blockade diminishes oxidative stress, inflammation and fibrosis, although it is not yet clear whether this relates to direct effects in the heart (mediated via fibroblasts) and/or

indirect peripheral effects (mediated via oxidative stress-activated mononuclear cells) [22].

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Nederlandse Samenvatting

Introductie en doelstelling (Hoofdstuk 1)

Van oudsher wordt aldosteron gezien als een steroïd uit de bijnier dat in de nieren werkt door activatie van intracellulaire mineralocorticoïde receptoren (MR). Angiotensine (Ang) II, hetzij lokaal in de bijnier gevormd of aangevoerd via de bloedbaan, is één van de factoren die de aldosteron synthese stimuleert. Twee recente klinische studies hebben gunstige effecten van MR antagonisten aangetoond bij patiënten met hartfalen, en dit kon niet alleen worden verklaard door blokkade van de renale MR-gemediëerde effecten op de bloeddruk. Er wordt nu verondersteld dat aldosteron ook effecten heeft buiten de nieren.

Dit hoofdstuk geeft een overzicht van de recente bevindingen op dit terrein en beschrijft ook de hypothese dat aldosteron extrarenale effecten heeft via een nieuwe (tot dusver niet-geïdentificeerde) membraan-gebonden receptor. Een onderscheid wordt gemaakt tussen de klassieke 'genomische' (bewerkstelligd na uren, en afhankelijk van *de novo* eiwitsynthese) en de nieuwe 'niet-genomische' (bewerkstelligd binnen minuten, zonder *de novo* eiwitsynthese) effecten van aldosteron. Een controversieel vraagstuk is tevens of aldosteron synthese ook buiten de bijnier geschiedt, bijvoorbeeld in het hart. Expressie van het belangrijke enzym aldosteron synthase is bijvoorbeeld aangetoond in verschillende cellen buiten de bijnier, inclusief die van het hart.

De doelstelling van de huidige studie is ten eerste te ontrafelen in hoeverre lokaal of uit bloed afkomstig Ang II de aldosteron synthese in de bijnier stimuleert, en tevens vast te stellen of de lokale produktie van Ang II in de bijnier intra- of extracellulair plaatsvindt. De genomische en niet-genomische effecten van aldosteron werden vervolgens bestudeerd in het hart van de rat, zowel onder normale als ischemische condities, en ook werd de bron van cardiaal aldosteron onderzocht. Tevens werden de effecten van aldosteron in humane myocardiale trabekels en coronaire arteriën gekwantificeerd, evenals de betrokken second messengers. Tenslotte werd aldosteron gemeten in normale en falende humane harten, en is bekeken of er een associatie is tussen het aldosteron synthase gen (CYP11B2) C-344T polymorfisme en de mate van cardiale hypertrofie bij patiënten met hypertrofe cardiomyopathie.

Oorsprong en plaats van vorming van Ang II in de bijnier (Hoofdstukken 2 en 3)

Infusie van [^{125}I]-gelabelde angiotensines bij varkens liet zien dat circulerend [^{125}I]-Ang II, maar niet circulerend [^{125}I]-Ang I, zich ophoopt in de bijnier. Tijdens steady-state is de [^{125}I]-Ang II spiegel in de bijnier 15-20 keer hoger dan in bloed. Echter, omdat de hoeveelheid endogeen Ang II in de bijnier ongeveer 400 keer hoger is dan die in bloed, wordt het overgrote deel van bijnier-Ang II toch lokaal in de bijnier gesynthetiseerd, vanuit lokaal gevormd Ang I. De Ang II type 1 (AT_1) receptor antagonist eprosartan reduceerde de ^{125}I-Ang II ophoping in de bijnier fors, en deed de Ang II concentratie in bloed sterker stijgen dan die in de bijnier. Als gevolg hiervan reduceerde eprosartan de weefsel/plasma Ang II concentratie ratio even sterk voor endogeen Ang II als voor ^{125}I-Ang II.

Hieruit kan worden geconcludeerd dat de vorming van Ang II in de bijnier extracellulair geschiedt, en wordt gevolgd door internalisatie via AT_1 receptor-gemediëerde endocytose. Kennelijk heeft het getrunceerde prorenine dat intracellulair voorkomt in bijnierweefsel (en dat volledig enzymatisch actief is), een andere functie dan Ang I vorming. Deze conclusie klopt ook met het feit dat cellen die angiotensinogeen maken dit meteen uitscheiden zonder het intracellulair op te slaan.

Effecten van aldosteron in het rattehart (Hoofdstukken 4 en 5)

In het geïsoleerde rattehart, geperfuseerd volgens Langendorff, verhoogde aldosteron de linker ventrikel druk en verlaagde het de coronairflow. Deze effecten zijn vergelijkbaar met die van Ang II. De MR antagonisten spironolacton and eplerenon blokkeerden de effecten van aldosteron niet. Spironolacton veroorzaakte zelfs vergelijkbare inotrope effecten, en het lijkt er dus op dat de effecten van aldosteron op flow en inotropie niet-genomisch van aard zijn.

Zowel spironolacton als eplerenon verbeterden de hartconditie na ischemie en reperfusie (infarctgrootte ↓, herstel linker ventrikel druk ↑, optreden van aritmieën ↓), hetgeen suggereert dat deze stoffen met de schadelijke effecten van endogeen aldosteron interfereren. Perfusiestudies met aldosteron lieten zien dat het steroïd zich snel ophoopt in het hart, zowel in de extracellulaire (interstitiële) ruimte als in een tweede, nog niet-

geïdentificeerd compartiment. Het uitwassen vanuit dit tweede compartiment gebeurde relatief snel (halfwaardetijd < 10 minuten), en na langdurig wassen lagen de aldosteron concentraties in het hart rond de detectielimiet. Dit betekent dat het grootste deel van het cardiale aldosteron uit bloed afkomstig is.

In gladde spiercellen uit de aorta bleken lage aldosteron concentraties het effect van Ang II op DNA synthese te versterken. Hogere aldosteron concentraties verlaagden juist de DNA synthese, zowel in gladde spiercellen als in cardiomyocyten. Hoge aldosteron concentraties reduceerden ook de collageen synthese in cardiofibroblasten. Spironolacton blokkeerde deze effecten van aldosteron, hetgeen suggereert dat de effecten op de synthese van DNA en collageen gemediëerd worden door MR en dus genomisch van aard zijn.

Samenvattend kan gesteld worden dat aldosteron positief inotrope en vasoconstrictoire effecten induceert in het rattehart via een niet-genomisch mechanisme, en dat deze effecten vergelijkbaar zijn met die van Ang II. MR stimulatie verlaagt de DNA en collageen synthese in hartcellen en verslechtert de conditie van het hart na het afsluiten van coronaire arteriën. Blokkade van deze laatste fenomenen zou ten grondslag kunnen liggen aan (een deel van) de gunstige effecten van MR antagonisten bij hartfalen.

Effecten van aldosteron in het humane hart (Hoofdstukken 6 en 7)

Aldosteron induceerde een negatief inotroop effect in humane myocardiale trabekels, in tegenstelling tot de positief inotrope effecten die eerder werden gezien in het rattehart. De proteïne kinase C (PKC) remmer chelerythrine chloride, maar niet de MR antagonisten spironolacton en eplerenon, blokkeerde het negatief inotrope effect. Kennelijk wordt het dus gemedieerd via een PKC-afhankelijk, MR-onafhankelijk mechanisme. De aldosteron concentraties die nodig waren om het negatief inotrope effect te induceren waren hoog en kwamen alleen voor in falende harten.

De aldosteron concentraties in het hart correleerden met die van renine, terwijl bekend is dat cardiaal renine uit de nieren afkomstig is. Het is daarom niet onwaarschijnlijk dat ook cardiaal aldosteron haar oorsprong heeft buiten het hart. Ondersteuning van dit concept wordt verkregen uit onze bevinding dat de cardiale en plasma concentraties van aldosteron bij patiënten met hypertrofe cardiomyopathie

identiek waren aan die van gezonde personen, ondanks het feit dat het aldosteron synthase gen (CYP11B2) C-344T polymorfisme geassocieerd is met de mate van cardiale hypertrofie bij deze patiënten. De meest voor de hand liggende verklaring van deze bevinding is dat de T allel-gerelateerde toename in plasma aldosteron de onderliggende reden is voor de relatie tussen het T-allel en cardiale hypertrofie, waarschijnlijk omdat deze leidt tot een parallelle verhoging van de cardiale aldosteron concentraties.

Aldosteron zelf had geen effect in humane coronaire arteriën. Voorafgaande blootstelling aan aldosteron verdubbelde echter de constrictoire respons op Ang II, waarschijnlijk door inductie van ERK1/2 fosforylering. Hydrocortison en 17β-estradiol potentieerden Ang II eveneens, maar daarvoor waren veel hogere (niet-fysiologische) concentraties nodig.

Concluderend induceert aldosteron een negatief inotroop effect in humane trabekels (daarmee het bekende positief inotrope effect van Ang II tegengaand) en potentieert het de vasoconstrictoire effecten van Ang II in coronaire arteriën. Deze effecten zijn non-genomisch van aard, en verlopen respectievelijk via PKC en ERK 1/2.

摘要

肾素-血管紧张素-醛固酮系統不仅是一个循环內分泌系统，而且存在于许多局部组织，如血管，心，脑，肾，肾上腺等组织，以旁分泌，自分泌或/和细胞內分泌的方式调节器官，组织或细胞的功能和结构。长期以來，治疗高血压和心力衰竭着重于拮抗肾素-血管紧张素系統而非肾素-血管紧张素-醛固酮系統。研究发现在使用血管紧张素转换酶抑制剂(ACEI)或血管紧張素II受体(AT1)拮抗剂时，醛固酮的合成并沒有被阻止，这个现象被称为肾上腺重新激活或醛固酮逃逸现象。醛固酮拮抗剂被用于治疗高血压已有多年的历史，近年来两个大型的临床实验(RALES和EPHESUS)证明醛固酮受体拮抗剂降低心功能不全患者的死亡率。目前已知醛固酮的这个作用完全独立于血管紧张转换酶抑制剂或血管紧张素拮抗剂的治疗作用，但具体的机理尚不清楚。探讨醛固酮详细的治疗作用机理成为近年来研究的热点。

本文第一章节在概述肾素-血管紧张素-醛固酮系統的基础上，全面介绍了醛固酮的生理，生化，药理学特性，心血管系统的病理作用以及与血管紧张素II的相互作用。局部血管紧张素的合成在很大程度上，如果不是全部的话，依赖于肾脏合成的肾素。但血管紧张素的合成究竟是在细胞外液，细胞表面，或者是在细胞的间隔还存在着争论。因此，在本论文第二章节我们综述了近年来对局部组织肾素-血管紧张素-醛固酮系统的研究，包括肾素，血管紧张素原，血管紧张素转换酶，血管紧张素及血管紧张素受体的研究，侧重于考虑细胞间是否存在肾素-血管紧张素系统的各个成分，包括前肾素原，肾素，血管紧张素，血管紧张素转换酶抑制剂，血管紧张素受体，以及局部前肾素原，肾素的激活，另外，我们还比较了研究所采用的动物模型(在体和离体)的不同性和不同研究方法的局限性，尤其是测量血管紧张素的难度，并最终得出结论:组织血管紧张素的合成是发生在细胞外，严格的来讲，是发生在细胞膜表面。

局部血管紧张素I的合成主要是依赖于肾源性的肾素，而局部的血管紧张素II则大部分是由局部的血管紧张素I所合成的。循环的血管紧张素II主要是在肾脏与其受体结合并聚集。本文第三章节使用动物模型猪研究了血管紧张素在肾脏的产生和合成部位(细胞内或细胞外)。通过测量和比较对照组，开搏通，洛沙坦治疗组血液/肾脏组织内源性和外源性的(经静脉注射^{125}I标记)血管紧张素I血管紧张素II的浓度，以及血管紧张素II/血管紧张素I的比率，研究结果表明90%的肾血管紧張素II来源于

肾脏。肾性血管紧張素II的合成发生于细胞外并随之通过其位于细胞膜上的受体内膜化。

本论文第四章节侧重于研究醛固酮对心血管系统的作用并进一步探讨醛固酮受体拮抗剂降低心衰患者死亡率的机理。在大鼠离体心脏，本研究观察到醛固酮具有正性肌力和收缩冠脉血管的作用。这些作用发生在数分钟(nongenomi-effects)且不能被安体舒通所阻断，其作用机理不同于众所周知的醛固酮通过激活其核受体所产生的保钠储水的作用(genomic-effects)。由此推测可能在细胞膜上存在一种尚为被人们确认和分类的新的醛固酮受体。在缺血-再灌注的大鼠离体心脏，本研究观察到醛固酮受体拮抗剂安体舒通产生似心肌缺血预适应的心肌保护作用。在培养的心肌细胞，血管平滑肌细胞和心肌成纤维细胞，本研究观察比较了醛固酮和血管紧張素II对DNA及胶原合成的作用。虽然醛固酮本身并无促生长的作用，但醛固酮具有增强血管紧張素II的促生长的作用。

本文第五章详细观察了心源性醛固酮的摄取和释放的药理动力学。醛固酮灌注离体大鼠心脏的试验发现醛固酮快速的堆积在心肌组织，这蓄积不但发生在细胞间，而且还发生在尚未被阐明的组织间隔。在冲洗时，醛固酮消失的半衰期是10分钟，随着长时间的冲洗，醛固酮水平不再能被检测的到，表明大部分心脏组织的醛固酮来源于血液循环。另外，新一代的醛固酮受体拮抗剂Eplerenone同样具有安体舒酮的似心肌缺血预适应的心肌保护作用，其作用机理可能与其抗心率失常的作用有关。

在人离体心脏(第六章节)本文研究表明血管紧张素II具有正性肌力的作用，而醛固酮具有负性肌力的作用。虽然醛固酮本身对人离体冠脉并无作用，但醛固酮明显增强血管紧张素II的收缩冠脉血管的效应。进一步研究表明醛固酮的负性肌力是通过蛋白激酶做为第二信息传导的，而醛固酮的促血管紧张素II的冠脉收缩作用是通过磷酸化ERK1/2做为第二信息传导的。上述的醛固酮作用因其反应快且不被醛固酮受体拮抗剂所阻断，也应归类为醛固酮的non-genomic效应。由此推测，血循环醛固酮水平的增高，进一步使心衰患者的冠状动脉循环恶化。

在本文的第七章节，研究不但比较了健康和心衰患者离体心肌组织的肾素及醛固酮水平，而且还观察了醛固酮合成基因片段多态性和肥厚性心肌病患者左心室

肥厚的相关性。结果显示心脏组织的肾素和醛固酮均来源于心脏外组织(血液循环)。醛固酮合成基因C-344T片段的多态性和肥厚性心肌病患者，尤其是男性的左心室肥厚的程度有很强的相关性。其可能的机理是C-344T等位基因导致血循环醛固酮水平升高，从而导致左心室肥厚。醛固酮拮抗剂是否能被用于治疗肥厚性心肌病，尤其是防治此类患者心性猝死，已引起临床工作者的浓厚兴趣。

纵上所述，醛固酮是肾素-血管紧张素-醛固酮系统的最终产物，其在心血管疾病如高血压，冠状动脉性心脏病，心力衰竭，及左心室肥厚的发生和发展中起很重要的作用，尤其是醛固酮和血管紧张素II对冠脉收缩的协同作用，可能导致心力衰竭患者的冠脉循环进一部恶化。醛固酮受体拮抗剂治疗高血压的效应已被广泛确认，其降低心力衰竭患者病死率也已被临床试验所证实，但是否能被用于治疗肥厚性心肌病并预防心性猝死，尚有待于进一部研究证实。

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Curriculum Vitae

Wenxia Chai was born on 5th February 1965 in Wanrong, Shanxi, P.R. China. In 1981, she enrolled in the Shanxi Medical University for medical study. After graduating as a medical doctor in 1986, she worked as a resident physician at the first academic hospital of Shanxi Medical University. In 1993, she followed the Master postgraduate programme and specialist training in Cardiology Department at the same hospital. In 1996, she started working as an attending physician in Cardiology department. In 1999, she came to The Netherlands and worked as a visitor scholar in Experimental Cardiology of Erasmus University Rotterdam, which was sponsored by the Ministry of Education (P. R. China) and the Netherlands University Foundation for international Co-operation (Nuffic). In 2000, she worked as a Ph. D candidate in Cardiovascular Pharmacology Department of Erasmus University Rotterdam. Since the end of 2005, she has been working as a Post-Doctoral Fellow at the interuniversity cardiology institute of the Netherlands, which belong to Royal Netherlands Academy of Arts and Sciences.