Molecular Mechanisms of Mineralocorticoid Receptor Antagonism by Eplerenone

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Abstract: Mineralocorticoid receptor (MR) antagonism has proven to effectively attenuate the pathophysiological effects of aldosterone in clinical and experimental settings of hypertension and heart failure. MR activates transcription of target genes upon aldosterone binding, and eplerenone selectively binds to MR and blocks aldosterone- mediated activation. In this review, we summarize the preclinical and clinical evidence supporting the beneficial effects of eplerenone (INSPRA™), a selective aldosterone blocker, in the treatment of hypertension and heart failure. We also review the current status in understanding the molecular mechanisms of action of the MR and its ligand. In addition, we compare the effects of eplerenone and spironolactone, a nonselective aldosterone blocker, on the transcriptional activity of MR and provide a molecular explanation for the improved side-effect profile of eplerenone compared with spironolactone.

THE ROLE OF MR AND ALDOSTERONE IN HYPERTENSION AND HEART FAILURE

Hypertension is a major risk factor for many common causes of morbidity and mortality including heart failure, myocardial infarction, stroke, and end-stage renal disease. Blood pressure is tightly linked to salt and water homeostasis, and molecular genetic studies indicate that almost all identified human mutations affecting blood pressure alter renal salt reabsorption [1]. Normal regulation of salt and water homeostasis in mammals requires an integrated network of control systems including the renin-angiotensin-aldosterone system (RAAS). Renin, which is secreted into the lumen of renal afferent arterioles by juxtaglomerular cells, cleaves angiotensinogen to form angiotensin I. Angiotensin-converting enzyme (ACE) subsequently cleaves angiotensin I to form angiotensin II (AII). Angiotensin II serves as a pivotal modulator of blood pressure in part by stimulating the synthesis and secretion of aldosterone by adrenal zona glomerulosa cells. Once released into the circulation, aldosterone promotes renal sodium reabsorption and potassium secretion in the distal nephron, distal colon, and salivary and sweat glands. Reabsorption of sodium and water then elevates blood pressure indirectly by expanding intravascular volume.

Because of the importance of the RAAS in hypertension, this system has been targeted heavily for therapeutic intervention. For example, common agents to treat hypertension included ACE inhibitors (ACEi), which block the conversion of the inactive angiotensin I to the active vasoconstrictor AII, and angiotensin receptor blockers (ARBs), which block the AII type I receptors, the isoform of the AII receptor known to mediate the primary pathophysiological effects of AII. Although ACEi and ARBs suppress aldosterone production, plasma aldosterone levels usually rise over time during chronic treatment in patients with hypertension and heart failure—a phenomenon called "aldosterone escape" [2]. This effect occurs even when patients receive maximal doses of both ACEi and ARBs [3]. Thus, patients treated with this standard of care remain unprotected from the effects of inappropriate levels of circulating aldosterone. How then can these patients be treated effectively? The solution may lie downstream of the RAAS – in blocking the deleterious effects of excess aldosterone.

Aldosterone exerts its effects through binding to the mineralocorticoid receptor (MR; NR3C2) and regulating gene expression. The MR has been identified in the kidney, heart, brain, and vasculature. In the kidney, the MR is primarily expressed in epithelial cells of the distal nephron. Here, aldosterone plays a pivotal role in regulating salt and water balance by regulating sodium, potassium, and hydrogen transport across epithelia. Consistent with the expression pattern of MR [4], abundant evidence now suggests that inappropriate levels of aldosterone, in the presence of moderate to high salt, can mediate significant damage in nonepithelial tissues including the heart, brain, and vasculature [5]. Furthermore, the enzymes responsible for aldosterone biosynthesis have also been identified in the same tissues [6-8]. Thus, the dogma around aldosterone has shifted from a steroid hormone mainly responsible for maintaining salt and water balance to a more ubiquitous effector hormone with demonstrated effects in multiple target organs and systems.

The first evidence that aldosterone plays an important role in nonepithelial tissues was reported by Brilla and Weber [9], who reported that elevated levels of aldosterone, in combination with a high-salt diet, induced cardiac hypertrophy and fibrosis in uninephrectomized rats. These effects were blocked by administration of MR antagonists. Subsequently, similar studies have confirmed these results.[10]. More recent studies in rats administered AII and the nitric oxide inhibitor *N*–-nitro-L-arginine methyl ester (L-NAME) demonstrated that marked cardiac and renal tissue and vascular damage resulting from elevated RAAS can be effectively attenuated by adrenalectomy [11]. These findings

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suggest that aldosterone is a primary mediator of AII-driven organ damage. Further studies showed that aldosterone induces coronary inflammatory lesions, which were preceded by, and associated with, the induction of cyclooxygenase-2 (COX-2), macrophage chemoattractant protein-1 (MCP-1), and osteopontin [12]. However, compelling evidence that aldosterone plays a deleterious role in the cardiovascular system is also provided by the Randomized Aldactone Evaluation Study (RALES) trial in late-stage heart failure patients. In this trial, spironolactone decreased mortality by 30% in patients with severe heart failure [13].

In the brain, high levels of MR are found in the hippocampus and intracerebroventricular (ICV) infusion of aldosterone significantly elevates blood pressure [14]. Moreover, ICV infusion of a selective mineralocorticoid antagonist, at doses that are ineffective when administered systemically, inhibits the development of the hypertension produced by the subcutaneous infusion of aldosterone or deoxycorticosterone in normotensive rats [15-17]. These data suggest that hypertensive effects of aldosterone are likely mediated, in part, by actions of this mineralocorticoid in the brain.

Aldosterone has been shown to play a major role in the development of renal vascular as well as cerebral vascular injuries in genetically hypertensive rats [11]. In recent studies, saline-drinking, uninephrectomized rats receiving exogenous aldosterone developed severe albuminuria and renal vascular injury. In addition, severe vascular and glomerular sclerosis characterized by fibrinoid necrosis and inflammation of the small and medium-sized renal arteries also was evident in these animals. Moreover, the expression of proinflammatory markers such as osteopontin, MCP-1, interleukin (IL)-1β, and IL-6 was increased by aldosterone [18]. Similar vascular damage induced by aldosterone also was observed in the brain in saline-drinking, spontaneously hypertensive stroke-prone rats. These results suggest that, as in the heart, vascular inflammation induced by aldosterone and dietary salt plays an important role in the development of vascular disease. Overall, numerous studies have shown that aldosterone exposure in the presence of moderate-to-high salt intake has deleterious outcomes that contribute to the pathogenesis of cardiovascular disease, which include myocardial fibrosis, thrombogenesis, vascular inflammation, and endothelial dysfunction. A detailed discussion of these topics is beyond the scope of this review and can be found in other reviews [19-21].

THE ANTIDOTE: EPLERENONE

The non-selective aldosterone antagonist. spironolactone, was developed by Dr. John Cella of Searle in the 1950's by combining the structure of progesterone to antagonize aldosterone and digitoxin to improve cardiac function (Fig. **1**), [22,23].

Subsequent studies in experimental models of cardiac damage established the cardioprotective benefit of spironolactone, which was confirmed in initial clinical studies in patients with heart failure [24]. Since the launch of spironolactone (Aldactone®) in 1960, this agent has been used to treat hypertension and heart failure. Although it is very effective in treating these diseases, spironolactone causes unwanted progestational and antiandrogenic side effects that limit its use. Eplerenone, a more selective MR antagonist, was developed to expand the utility of aldosterone blockade as a result of a superior side-effect profile. Numerous preclinical and clinical studies have demonstrated the efficacy of eplerenone for the treatment of hypertension and heart failure, and these findings are summarized below.

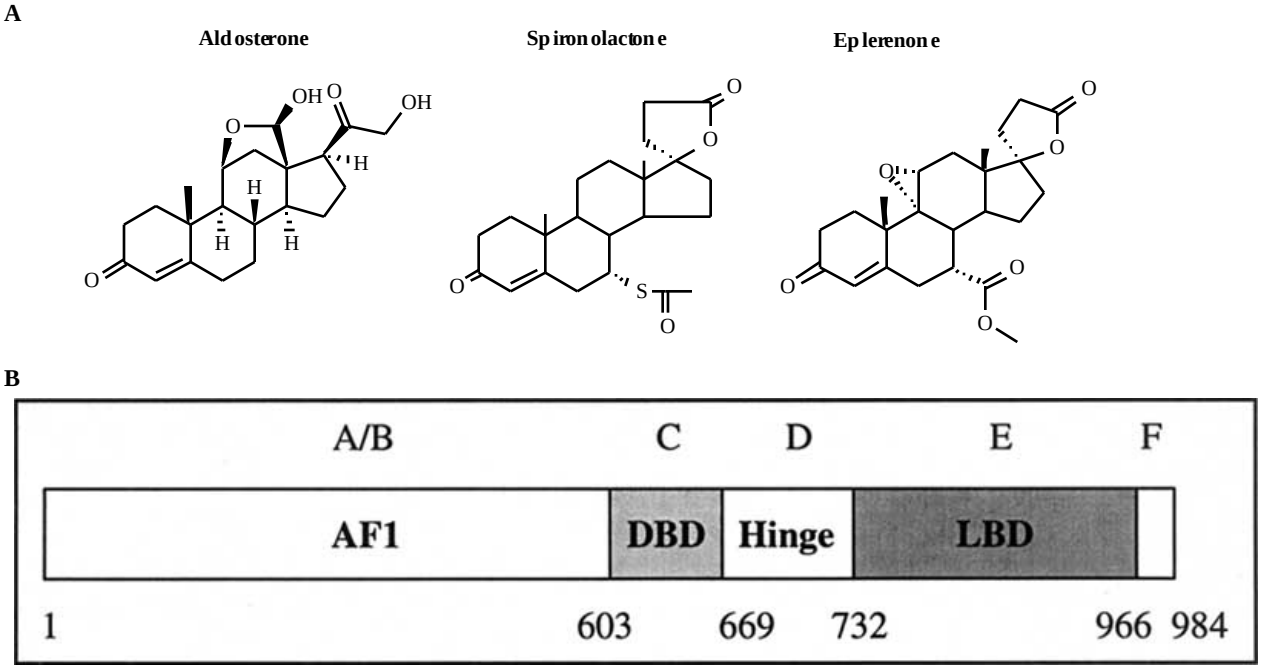

Fig. (1). A. Structures of the MR agonist aldosterone and the antagonists spironolactone and eplerenone. B. Schematic representation of the MR subdomain structure. The letters (A-F) on the top represent the original domain names. The functional domains are labeled within the boxes. The numbers on the bottom represent the residue numbers in MR protein.

Antihypertensive Efficacy of Eplerenone

In the Kagawa assay in which inhibition of aldosterone-mediated decreases in urinary Na^+/K^+ ratios are measured, eplerenone effectively blocks the reabsorption of Na^+ and decreases the excretion of K^+, thereby increasing urinary Na^+/K^+ ratios. In this assay, eplerenone is nearly equipotent compared with spironolactone despite a 20-fold lower affinity for MR compared with spironolactone. This discrepancy is thought to arise from lower plasma protein binding of eplerenone [25,26]. In addition, eplerenone produces significant blood pressure lowering in several animal models of hypertension. For example, oral administration of eplerenone significantly attenuated the progressive increase in systolic blood pressure in aldosterone/salt-treated rats [12,18]. Moreover, overstimulation of MR by glucocorticoids via inhibiting the glucocorticoid-converting enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) leads to severe hypertension in Wistar rats, and treatment with eplerenone for 2 weeks normalized blood pressure [27].

In clinical trials, eplerenone also has been demonstrated to effectively lower blood pressure in hypertensive patients. One study comparing different doses of eplerenone with spironolactone demonstrated a dose-dependent reduction of blood pressure. For example, at 100 mg, eplerenone reduced blood pressure by 75% compared with spironolactone (100 mg), and eplerenone efficacy was associated with an improved side-effect profile compared with spironolactone [28]. Several double-bind, titration-to-effect clinical studies also have been conducted to compare the antihypertensive effects of eplerenone to standard therapies including an ACEi (enalapril), an ARB (losartan), and a calcium-channel blocker (amlodipine) [29-32]. In general, eplerenone was at least as effective as these antihypertension therapies in controlling blood pressure, with fewer side effects, such as cough and edema. Moreover, in hypertensive patients whose blood pressure was not controlled by an ACEi or an ARB, eplerenone demonstrated a further reduction in blood pressure compared with monotherapy, suggesting that eplerenone is useful as add-on therapy when blood pressure cannot be controlled by these agents [33].

Protective Effects of Eplerenone on the Heart

Numerous animal and clinical studies have demonstrated that eplerenone is a beneficial agent in the treatment of heart failure. In Sprague-Dawley rats, eplerenone treatment protects against maladaptive remodeling after myocardial infarction without significantly impacting infarct healing [34]. In dogs with heart failure, chronic treatment with eplerenone attenuated progressive cardiac dysfunction and left ventricular remodeling without affecting systemic blood pressure, heart rate, or systemic vascular resistance [35]. As discussed earlier, rats receiving salt, AII, and L-NAME develop hypertension, which is accompanied by extensive cardiovascular damage. Eplerenone treatment or removal of the primary source of endogenous aldosterone through adrenalectomy did not significantly reduce blood pressure in these rats. However, histological studies of the hearts revealed that cardiac and renal injury was markedly reduced in adrenalectomized animals and those receiving chronic administration of eplerenone [12].

The recently completed Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) trial was designed to investigate the efficacy of eplerenone in patients with acute myocardial infarction with evidence of left ventricular dysfunction and heart failure. Patients received either placebo or eplerenone (43-mg/d average dose) in addition to standard therapy. The study continued until 1012 deaths occurred. The results of this study were published recently [36]. The overall mortality decreased 15% ($P<0.008$) with eplerenone treatment. Death from cardiovascular causes or hospitalization for cardiovascular events were reduced 13% ($P<0.002$) by eplerenone. Several other secondary endpoints including sudden death from cardiac causes and episodes of hospitalization for cardiovascular events also were significantly reduced. Thus, the addition of eplerenone to optimal medical therapy results in improved survival and reduced hospitalization among patients with acute myocardial infarction complicated by left ventricular dysfunction and heart failure.

Protection of the Kidney by Eplerenone

In animal models, aldosterone/salt-induced albuminuria and renal vascular injury were significantly reduced by eplerenone [18]. Similarly, in clinical trials, eplerenone reduced microalbuminuria as effectively as an ACEi (enalapril) and an ARB (losartan), and much better than a calcium-channel blocker (amlodipine) [30-32]. Eplerenone also reduced the urinary albumin:creatinine ratio in diabetic hypertensive patients to a greater extent compared with enalapril [37]. In this case, renal protection was independent of significant blood pressure reduction, suggesting that eplerenone may provide renal protection in hypertensive patients with type 2 diabetes.

MOLECULAR MECHANISM OF ACTION

MR is a member of the nuclear receptor superfamily [38], which consists of about 48 proteins in humans. MR belongs to the steroid receptor subfamily, which also includes the androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), and progesterone receptor (PR). Similar to other nuclear receptors, MR can be divided into several discrete domains (Fig. **1B**). MR has a long N-terminal A/B region (1-602) that harbors activation functions (AF1). This is followed by a highly conserved DNA binding domain (DBD) and a less conserved ligand binding domain (LBD). A variable hinge region (D region) connects the DBD (C region) and the LBD (E region). At the C-terminal end, a small F region follows the LBD. Among all the known nuclear receptors, MR is most similar to GR, PR and AR. For example, in the DBD, MR is 91%, 90% and 77% identical to GR, PR and AR, respectively. In the LBD, the sequence identity is 56%, 55% and 51%, respectively.

The N-terminal A/B regions of nuclear receptors differ substantially in length. In general, this region of the nuclear receptor contains ligand-a independent activation function—the so-called AF1 domain. In MR, 2 independent AF1 domains have been identified [39]. They are termed AF1a and AF1b. Recently, a CBP-containing histone acetyltransferase (HAT) complex has been shown to interact

with AF1a [40]. The HAT complex is recruited to AF1a of MR via a direct binding of RNA helicase A (RHA) to MR AF1a. Histone acetylation has been linked to transcriptional activation on the chromatin template. It is interesting to note that the LBD of nuclear receptors also recruit HAT complexes (see below). However, the exact role of RNA helicase and the HAT complex on the overall transcriptional activity of MR remains to be elucidated. It is also interesting to point out that the N-terminal of MR interacts with the LBD in an agonist-dependent manner and that this interaction is blocked by antagonists of MR [41].

The DBD of the nuclear receptors contains two zinc fingers. Crystal structures of several receptor DBD complexed with DNA have been reported [42-44]. In general, the DBD binds to DNA as a dimer, and a dimer interface lies within the second zinc finger. A recognition helix, also called P-box, follows the first zinc finger making specific contacts with the major groove of the DNA double helix. The P-box sequences of MR are identical to those of GR, PR, and AR. It is therefore not surprising that these receptors recognize identical DNA sequences, with a consensus palindromic element spaced by 3 nucleotides: AGAACAnnnTGTTCT [45]. To date, all the known MR response elements (MRE) also are responsive to GR, PR, and AR, and no MR-specific DNA binding element has been identified.

It is the LBD that binds ligand and relays signals to transcription machinery. The crystal structures of many nuclear receptor LBD have been solved. These include the LBDs of wild-type ER, PR, and AR, and mutant GRs [46-51]. The canonical structure of the nuclear receptor LBD consists of 11 to 12 helices, with helix 2 present only in some receptors. The last helix (H12), often referred to as AF2, plays a critical role in a ligand-induced transcription switch. Upon agonist binding, this helix packs toward the core structure to seal the ligand-binding pocket and provide part of the binding surface for coactivator proteins (coactivators and their interactions with the LBD are discussed below).

The importance of H12 also is higlighted by the antagonist-bound LBD structures. Upon antagonist binding, H12 is twisted to occupy the binding surface for coactivators, thus preventing coactivators from binding to the receptor [46]. The crystal structure of MR has not been solved. However, homology models based on other nuclear receptor structures suggest that the MR LBD adopts similar structures [52,53]. Several naturally occurring mutations have been identified in the LBD. One of these mutations, S810L, which causes early-onset hypertension, is located in helix 5 in the homology model. Why MR antagonists such as progesterone or spironolactone activate this mutant receptor is not clear, although it is proposed that this mutation creates an additional interaction with helix 3 to stabilize the activated structure [53]. X-ray crystallography and detailed mutagenesis studies are needed to answer this important question.

MR, like other steroid receptors, functions as a homodimer on DNA. The DBD contains a weak dimerization interface, as discussed earlier while the LBD harbors a second dimerization function. For other receptors, the dimerization function has been mapped to a region corresponding to helix 9 and helix 10 of the LBD structure. Recently, it has also been shown that MR and GR can form heterodimers on DNA [54-56]. However, the biological significance of this interaction remains to be discovered.

When in the nucleus, agonist-occupied nuclear receptors recruit coactivator proteins to increase transcription and a number of coactivator proteins have been identified [57]. These coactivator proteins interact with nuclear receptors via small peptide motifs called the NR box [58], which contains LXXLL motifs in an α-helical structure and binds the coactivator binding groove formed by helix 3-5 and helix 12 of the LBD [59]. Among these coactivators, 3 so-called p160 family coactivators (SRC1, GRIP1, and AIB1) have been studied extensively [60]. They share significant homology and contain HAT activities. Two of these coactivators, SRC1 and GRIP1, have been shown to interact with MR in an agonist-dependent manner [61,62]. Interestingly, SRC1 and GRIP1 also interact with CBP [63], which also contains HAT activity. As discussed earlier, the N-terminal A/B region of MR recruits a complex containing CBP [40]. Together with the finding that the A/B region also interacts with the LBD in an agonist-dependent manner [41], these results raise an interesting question of how these protein complexes coordinate to generate productive transcriptional activation. Answers to this question may also shed light on the role of the N-terminal AF1 in the overall transcriptional activity of MR. Moreover, the p160 family of coactivators are generally ubiquitously expressed and are recuited by most nuclear receptors. The roles of other, more tissue-specific coactivators such as PGC1 in MR activation remain to be defined [64,65].

In the absence of ligand, some nuclear receptors repress transcription by binding corepressors. Steroid receptors in general are not in the nucleus in the absence of ligand, and they do not interact with corepressors even if they are transported into nucleus by fusion of nuclear localization signals. However, under some conditions, antagonist-bound steroid receptors recruit corepressors. For example, tamoxifen-bound ER recruits corepressors on a chromatin template [66]. Two common corepressors, N-CoR and SMRT, have been identified for nuclear receptors [67]. These corepressors recruit histone deacetylases (HDACs) and interact with nuclear receptor via peptide motifs called CoRNR boxes [68]. In contrast to histone acetylation, histone deacetylation has been associated with transcription silencing. It would be very interesting to find out whether spironolactone or eplerenone bound MR could interact with corepressors under certain conditions. A detailed understanding of MR and its interactions with coactivators and corepressors in the presence of agonists and antagonists should help define the molecular mechanism by which MR antagonists decrease aldosterone activated transcription. This could occur by blocking coactviator interactions (passive antagonism) or by actively recruiting corepressors to inhibit transcription (active antagonism). Examples of both types of antagonists have been reported for other nuclear receptors [69].

Many nuclear receptors, especially those that heterodimerize with the retinoid X receptor (RXR), appear to be in the nucleus even before binding ligand. On the other

hand, MR, similar to other steroid receptors, is localized to the cytoplasm when unliganded [70]. In the cytoplasm, MR is bound to heat-shock proteins, such as hsp70, hsp90, or the immunophilin FKBP-52 [71-73]. Aldosterone binding dissociates MR from these proteins, and liganded MR is then translocated into the nucleus. In the nucleus, agonist-bound MR forms nuclear clusters [70]. Spironolactone also has been shown to translocate MR into nucleus, albeit at a slower rate. However, spironolactone does not induce nuclear cluster formation; instead, it dissociates the nuclear clusters induced by aldosterone. The function of these nuclear clusters is not clear, but it is speculated that they are probably present in transcriptionally active euchromatin, suggesting a link to transcription activation. Thus, MR antagonist inhibits transcription activation and breaks up clusters**.**

Once in the nucleus, MR functions as a transcription factor by binding to DNA elements (MRE) near target genes. MRE from several genes that are regulated by MR have been identified. The most extensively studied genes are serum and glucocorticoid-regulated kinase (SGK), epithelial sodium channel (ENaC), and Na^+/K^+ ATPase [74-77]. SGK is a serine/threonine kinase that is induced rapidly by both glucocorticoids and mineralocorticoids [78,79]. It has been shown that SGK plays an important role in regulating epithelial transporters [80,81]. For example, NEDD4 directly interacts with ENaC subunits and targets this channel for degradation [82,83], whereas SGK directly interacts with this ubiquitin ligase to block its functional binding to ENaC [84]. Thus, SGK increases the level of ENaC proteins in the membrane.

ENaC is composed of 3 subunits—α, β, and γ—and regulates reabsorbtion of Na^+ ions in the apical membrane of the cortical collecting tubule (CCT) in the distal nephron. Among all the MR target genes identified so far, ENaC is probably a key gene with relevance to aldosterone physiology and pathophysiology. Gain-of-function mutations in ENaC, which delete or mutate the cytoplasmic carboxyl termini of either the β or the γ subunit, cause early-onset hypertension, hypokalemic alkalosis, and low aldosterone levels—a disease called Liddle Syndrome [85,86]. Studies of patients with Liddle Syndrome indicate that these mutations increase the number of channels in the membrane by prolonging the half-life of ENaC [87]. Thus, Liddle Syndrome probably results from the inability to clear ENaC from the membrane.

In contrast, loss-of-function mutations in ENaC cause salt wasting, hypotension, and hyperkalemia in patients, despite high levels of aldosterone—a disease called recessive pseudohypoaldosteronism type I (PHAI). Patients with PHAI have impaired ENaC function [88,89], resulting in severe salt wasting. An MRE has been identified from the promoter of the α subunit of ENaC [75]. This MRE is responsive to both MR and GR. The existence of dual control mechanisms by which ENaC gene expression is regulated by MR, and the ENaC protein level in the membrane is regulated by SGK, which highlights the importance of ENaC as an effector of MR function in salt balance. However, despite the importance of ENaC in hypertension pathophysiology, it is intriguing that the distal tubule, where ENaC mainly reside, is only responsible for 2% of the salt reabsorption [1].

Na^+/K^+ ATPase also has been identified as an aldosterone target and is an integral membrane protein responsible for the active transport of sodium and potassium across the basolateral membrane in an ATP-dependent manner. Na^+/K^+- ATPase consists of 2 subunits—α and β—and MREs have been identified from the promoters of both subunits [76,77]. Net sodium reabsorption is the major function of renal Na^+/K^+-ATPase, and a close relationship exists between the abundance of Na^+/K^+- ATPase and the sodium reabsorption capacity of the different segments of the nephron [90].

Current literature also suggests a number of other genes as potential targets for aldosterone, including the recently identified inflammatory marker genes, osteopontin and MCP-1 [12]. However, whether or not they are direct MR target genes is not known. These inflammation genes are probably indirect target genes as their elevation in response to aldosterone only occurs after a prolonged treatment.

MR AND GR: FRIEND OR FOE?

MR and GR have a lot in common. *In vitro*, the mineralocorticoid aldosterone can bind to and activate both MR and GR, although a higher concentration of aldosterone is required to activate GR. Moreover, glucocorticoids, such as cortisol, also can bind to both receptors with MR having a higher affinity for cortisol than GR. Both receptors also share the same DNA response element sequences, and the target genes of one receptor can also be regulated by the other. To date, no MR-specific target genes have been identified. MR and GR also are colocalized in many tissues, including the cortical collecting duct, brain hippocampus, and arterial smooth muscle cells [91-93], and, in most cases, GR is more abundant than MR.

Given the similarity of MR and GR, one would imagine that they play similar physiological roles. However, this apparently is not the case. GR is involved in gluconeogenesis, anti-inflammation, and osteoporosis, whereas MR is involved in salt balance and is a potential driver of inflammation as well as tissue and vascular damage. *In vivo*, the circulating concentration of cortisol is up to 1000-fold higher than that of aldosterone, yet almost all MR activation is mediated by aldosterone [94]. Then, how does MR distinguish aldosterone from cortisol? Part of the answer lies in the enzyme 11β-HSD2.

11β-HSD2 converts cortisol (but not aldosterone) to its 11-keto metabolite cortisone, which does not activate MR [95]. In epithelial cells, including cells in the cortical collecting duct, 11β-HSD2 is expressed at high levels. The high levels of 11β-HSD2 protect MR from cortisol activation, thereby allowing aldosterone to bind to and activate MR [96,97]. When 11β-HSD2 is blocked by its inhibitor carbenoxolone corticosterone (rodent glucocorticoid) acts as a potent mineralocorticoid to increase sodium reabsorption [98]. The importance of 11β-HSD2 in protecting MR from abnormal activation by glucocorticoids also is exemplified by the syndrome of apparent mineralocorticoid excess (AME), which results from loss-of-

function mutations in the 11β-HSD2 gene [95]. Patients with AME have early-onset hypertension, hypokalemia, and suppressed plasma renin and aldosterone levels. In AME, the absence of 11β-HSD2 allows cortisol to activate MR, resulting in hypertension.

In most nonepithelial tissues, including the heart and the AV3V region of the brain, 11β-HSD2 is not coexpressed with MR. Thus, MR is unprotected. Unprotected MR is presumably occupied by the much higher level of circulating glucocorticoids. However, unlike in epithelial cells, in which cortisol activates MR, glucocorticoids antagonize MR in these nonepithelial cells. For example, central infusion of corticosterone into the brain blocks the hypertensive effects of aldosterone [15], and in the heart, coadministration of corticosterone inhibits cardiac hypertrophy and cardiac fibrosis induced by aldosterone [99].

Another piece of evidence demonstrating that MR appears to be protected in the heart from glucocorticoid occupation is the study on heart-specific 11β-HSD2–overexpressing transgenic mice [100]. Ectopic expression of 11β-HSD2 in the heart under the myosin heavy-chain promoter generates mice with cardiac hypertrophy, fibrosis, and heart failure. This phenotype can be suppressed by eplerenone administration, suggesting that inactivation of glucocorticoids in the heart leads to activation of MR. However, not all MR-expressing nonepithelial tissues are equal. In vascular smooth muscle cells (VSMC) and the amygdala region of the brain, MR appears to be protected against glucocorticoid occupation, because 11β-HSD2 is expressed at substantial levels [101]. In VSMC, inhibiting 11β-HSD2 by carbenoxolone has been shown to allow cortisol to activate MR, resulting in an inflammatory response in the vasculature [102].

The mechanism of the tissue-specific differences in the activity of glucocorticoids on MR is not clear. However, several recent studies have provided insight into this puzzle. As discussed earlier, the N-terminal region of MR recruits a HAT complex via RHA [40]. In the same study, it was shown using chromatin immunoprecipitation assays that RHA is recruited to MR target gene promoters in the presence of aldosterone, but not cortisol. Another clue also involves the N-terminal region of MR. As mentioned earlier, the N-terminal A/B domain interacts with the wild type and a mutant MR LBD in an aldosterone-dependent manner. Cortisol, on the other hand, promotes very weak interactions between the A/B domain and the mutant receptor LBD and blocks aldosterone-induced interaction, suggesting that aldosterone and cortisol are different in activating transcriptional activity of MR. However, there is one caveat with this result: This finding was demonstrated using a mutant form of the receptor. Thus, one alternative explanation is that this mutant has preference for different ligands in its interaction with the N-terminal region, a possibility that must be eliminated by studying wild-type receptors. Nonetheless, these differences may have important consequences for the activation function of the receptor in certain tissues. Together, these clues suggest that the N-terminal AF1 may be the mediator of tissue-specific effects of cortisol.

Moreover, MR and GR share extensive homology in the DBD and LBD, and recent studies using chimeras have mapped the sequences that determine the binding selectivity of aldosterone and cortisol [103]. A region on MR corresponding to helix 5–8 of the LBD is involved in high-affinity aldosterone binding, but not in high-affinity cortisol binding. In contrast, distinct sequences are involved in high-affinity cortisol binding. It will be very interesting to find out how these sequences communicate with other sequences involved in receptor activation and how these differences in binding could translate into tissue specificity.

Another interesting difference between MR and GR is their roles in inflammation and cardioprotection. Glucocorticoids have been used extensively as anti-inflammatory drugs. Agonist-bound GR inhibits the production of inflammatory cytokines, such as tumor necrosis factor α and IL-1, at least in part by repressing the action of transcription factor NF-κB and AP-1. GR also enhances the expression of annexin 1 to exert cardioprotective effects [104]. On the other hand, activated MR is proinflammatory and contributes to significant tissue and vascular injury. Specifically, aldosterone-bound MR has been shown to induce inflammatory markers such as osteopontin, MCP-1, and COX-2 in the media of coronary arteries, and eplerenone effectively attenuates the expression of these proinflammatory molecules [12]. Consistent with the notion that MR antagonists are anti-inflammatory, blocking MR activity by spironolactone has been shown to downregulate AP-1 and NF-κB [105].

SIDE EFFECTS OF MR ANTAGONISTS

As mentioned earlier, spironolactone has documented progestational and antiandrogenic side effects, which limit its use. In the RALES trial, for example, 10% of male patients given spironolactone with an average dose of 26 mg daily experienced gynecomastia or breast pain compared with 1.5% in the standard therapy group [13]. In addition, in hypertension trials, a dose-dependent increase of gynecomastia was observed. At doses of 50 mg or less, only 6.9% of male patients developed gynecomastia; the incidence was 13% at 100 mg and 52.2% for doses of 150 mg or higher [106]. In female patients, spironolactone treatment also has been shown to result in breast pain and irregular menstrual cycles.

The sex hormone–related side effects are most likely due to the ability of spironolactone to modulate AR and PR activities. This postulate has been confirmed both *in vitro* and *in vivo*. Binding assays using extracts from animals and radiolabeled ligand (rat kidney extracts and ^{3}H-aldosterone for MR, rat ventral prostate extracts and ^{3}H-methyltrienolone for AR, rabbit uteri extracts and ^{3}H-progesterone for PR, and rat kidney extracts and ^{3}H-dexamethasone for GR) showed that spironolactone binds MR with high affinity [25]. However, significant binding also was demonstrated at AR, PR, and to a lesser extent, GR. The potential for antiandrogenic and progestational effects of spironolactone also has been tested in intact male rats, castrated male rats receiving testosterone, estrogen-sensitized female rabbits, and cycling adult female rats [107,108]. These *in vivo* results confirm that spironolactone was antiandrogenic and antiovulatory. In contrast, in the same study, eplerenone

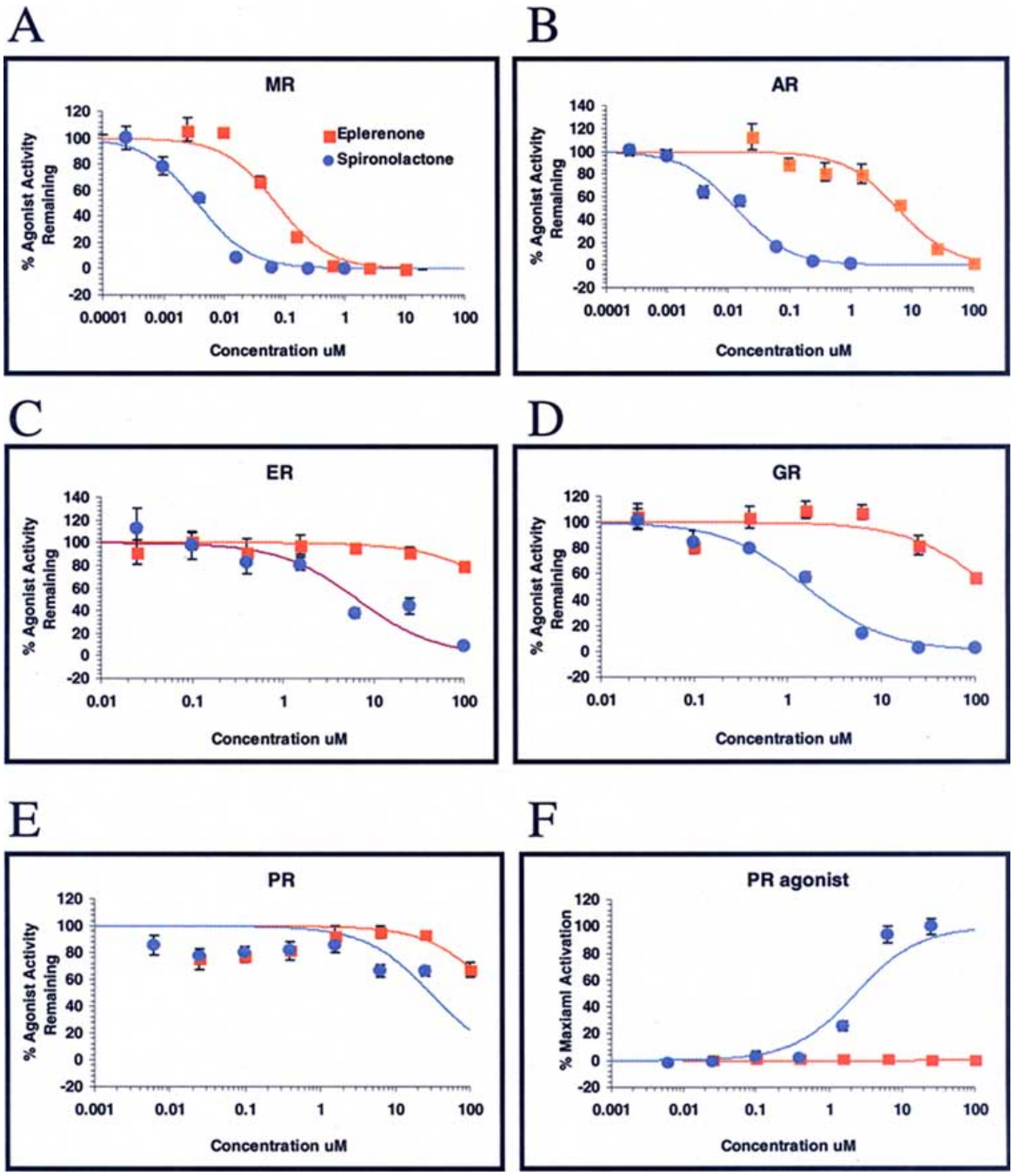

Fig. (2). Comparison of the effects of eplerenone and spironolactone on transcriptional activities of steroid receptors. A-E. Antagonist activity on MR, AR, ER, GR, and PR, respectively. F. PR agonist activity. Agonists used in the antagonist mode were: 0.5 nM aldosterone (MR), 10 nM dihydrotestosterone (AR), 5 nM dexamethasone (GR), 50 nM progesterone (PR) or 10 nM estradiol (ER) (all chemicals from Sigma, St. Louis, MO). Agonist concentrations represent 70%–80% of full activation of each receptor (2–3 times the EC_{50}). Cells were transfected with a luciferase reporter gene under the control of a GAL4 response element, along with a plasmid containing the Gal4 DNA-binding domain (DBD) fusion of a steroid receptor (MR, AR, GR, PR, and ER) LBD and a β-galactosidase control plasmid. Agonist of each receptor can bind to and activate the receptor LBD, which activate the expression of the Gal4 response element containing the luciferase reporter gene. Antagonists can compete for binding to the receptor LBD and decrease the transcription activity of the reporter gene. Measurement of luciferase activity allows quantitative determinations of the reporter transcription in the presence of either agonists or agonists and antagonists in combination. β-Galactosidase activity, which is unaffected by ligand, is used to normalize the transfection efficiency in the cell population. The data then were normalized as the percentage of full agonist activity remaining following addition of antagonist**.** The data reported are the mean of percent agonist activity remaining for the 6 replicate wells. For agonistic activity on PR, the raw data were normalized according to the maximal agonist activity and reported as a percentage of maximal activation. The smooth lines represent the fitted curves. The error bars represent standard errors.

displayed a lower potency compared with spironolactone in affecting testosterone target organs. Eplerenone was also devoid of any progestational effects and did not disturb ovulatory cycles. *In vitro* binding studies also indicated that eplerenone binds AR and PR with much lower affinity compared with spironolactone. Moreover, in the EPHESUS trial, eplerenone did not significantly increase the incidence of endocrine side effects. These adverse events were not significantly different between eplerenone and placebo groups in hypertension trials [33].

Table 1. Comparison of the Half Effective Concentrations (μM) of Eplerenone and Spironolactone

	Eplerenone	Spironolactone
MR (IC_{50})	0.0809	0.0024*
AR (IC_{50})	4.8265	0.0130*
GR (IC_{50})	>100	2.8994*
PR (IC_{50})	>100	>25
PR Agonist (EC_{50})	>100	2.6188*
ER (IC_{50})	>100	5.7015*

*P<0.05 vs eplerenone.

Half-effective concentration, the concentration of ligand to achieve 50% of the inhibition (IC_{50}) or 50% of the activation (EC_{50}).

The half-effective concentration numbers were obtained by curve fitting using the 4-parameter logistic model (y = (a-d)/(1+(x/c)^b)+d), with the lower (a) and upper (d) plateaus fixed at 0% and 100%, except for the PR antagonist mode, for which the lower plateau was not fixed. "b" is the slope; "c" is the IC_{50} or EC_{50}; "x" is the concentration of the compound; and "y" the activity at that concentration (percent activity remaining or percent maximal activation for antagonist mode or agonist mode, respectively).

To further explore the effects of MR antagonists on transcription and to compare the potency and specificity of eplerenone with spironolactone, we employed a cell-based luciferase reporter assay. This assay takes advantage of the fact that the LBD of nuclear receptors can respond to corresponding ligand and affect transcription when tethered to DNA by a heterologous DNA binding domain. The LBDs of steroid receptors were fused to the yeast transcription factor GAL4 DNA-binding domain and transfected into cells with a luciferase reporter under the control of a GAL4 response element. As shown in (Fig. **2**) and Table **1**, both eplerenone and spironolactone exhibit dose-dependent inhibition of MR transcriptional activity in the presence of 0.5 nM aldosterone. Consistent with the binding affinity, eplerenone appears to be less potent in antagonizing MR transcription activity compared with spironolactone. The IC_{50} of eplerenone for MR was 81 nM compared with 2.4 nM for spironolactone. Spironolactone also antagonized AR at low concentrations (IC_{50} = 13 nM). However, under the same conditions, a much higher concentration of eplerenone was required to inhibit 50% of AR transcriptional activity (IC_{50} = 4.8 μM). Thus, the potency of eplerenone at AR was reduced by ~370-fold compared with spironolactone.

Spironolactone also antagonizes GR and ER, albeit at high concentrations. IC_{50} values were 2.9 and 5.7 μM for GR and ER, respectively. In contrast, eplerenone did not appreciably antagonize GR or ER at concentrations up to 100 μM, which represents the highest concentration evaluated in the study. Thus, eplerenone exhibits much weaker activity at these receptors compared with spironolactone. In addition, spironolactone demonstrates limited antagonist activity at PR (IC_{50} >25 μM). However, in the absence of the PR agonist, spironolactone effectively activated PR with an EC_{50} of 2.6 μM. In sharp contrast, eplerenone neither activated nor antagonized PR activity at concentrations up to 100 μM. Taken together, these results indicate that eplerenone is less potent than spironolactone at MR, but more selective against other steroid receptors. Additionally, these findings provide insight into the molecular mechanisms of eplerenone selectivity [33,36].

SUMMARY

The extensive body of work presented represents decades of studies in both experimental and clinical settings. Taken together, these findings provide a molecular rationale for the superiority of eplerenone over spironolactone and establish eplerenone as an effective and selective aldosterone blocker for the treatment of hypertension and heart failure.

REFERENCES

[1] Lifton, R.P.; Gharavi, A.G.; Geller, D.S. *Cell*, **2001**, *104*, 545.
[2] Struthers, A.D. *Eur. Heart J.*, **1995**, *16* (Suppl. N), 103.
[3] Mckelvie, R.S.; Yusuf, S.; Ericak, D.; Burns, R.J.; Probstfield, M.; Tsuyuki, R.T.; White, M.; Rouleau, J.; Latini, R.; Maggioni, A.; Young, J.; Pogue, J. *Circulation*, **1999**, *100*, 1056.
[4] Lombes, M.; Oblin, M.E.; Gasc, J.M.; Baulieu, E.E.; Farman, N,; Bonvalet, J.P. *Circ. Res.*, **1992**, *71*, 503.
[5] Rudolph, A.E.; Rocha, A.; McMahon, E. *Mol. Cell Endocrinol.*, **2003**, In press.
[6] Silvestre, J.S.; Robert, V.; Heymes, C.; Aupetit-Faisant, B.; Mouas, C.; Moalic, J.M.; Swynghedauw, B.; Delcayre, C. *J. Biol. Chem.*, **1998**, *273*, 4883.
[7] Takeda, Y.; Miyamori, I.; Yoneda, T.; Iki, K.; Hatakeyama, H.; Blair, I.A.; Hsieh, F.Y.; Takeda, R. *Hypertension*, **1995**, *25*, 170.
[8] MacKenzie, S.M.; Clark, C.J.; Ingram, M.C.; Lai, M.; Seckl, J.; Gomez-Sanchez, C.E.; Fraser, R.; Connell, J.M.; Davies, E. *Endocr. Res.*, **2000**, *26*, 531.
[9] Brilla, C.G.; Pick, R.; Tan, L.B.; Janicki, J.S.; Weber, K.T. *Circ. Res.*, **1990**, *67*, 1355.
[10] Young, M.J.; Funder, J.W. *Am. J. Physiol.*, **1996**, *271*, E883.
[11] Rocha, R.; Stier, C.T., Jr.; Kifor, I.; Ochoa-Maya, M.R.; Rennke, H.G.; Williams, G.H.; Adler, G.K. *Endocrinology*, **2000**, *141*, 3871.
[12] Rocha, R.; Rudolph, A.E.; Frierdich, G.E.; Nachowiak, D.A.; Kekec, B.K.; Blomme, E.A.; McMahon, E.G.; Delyani, J.A. *Am. J. Physiol. Heart Circ. Physiol.*, **2002**, *283*, H1802.
[13] Pitt, B.; Zannad, F.; Remme, W.J.; Cody, R.; Castaigne, A.; Perez, A.; Palensky, J.; Wittes, J. *N. Engl. J. Med.*, **1999**, *341*, 709.
[14] Gomez-Sanchez, E.P. *Endocrinology*, **1986**, *118*, 819.
[15] Gomez-Sanchez, E.P.; Venkataraman, M.T.; Thwaites, D.; Fort, C. *Am. J. Physiol.*, **1990**, *258*, E649.
[16] Gomez-Sanchez, E.P.; Fort, C.M.; Gomez-Sanchez, C.E. *Am. J. Physiol.*, **1990**, *258*, E482.
[17] Gomez-Sanchez, E.P.; Fort, C.; Thwaites, D. *Am. J. Physiol.*, **1992**, *262*, E96.
[18] Blasi, E.R.; Rocha, R.; Rudolph, A.E.; Blomme, E.A.; Polly, M.L.; McMahon, E.G. *Kidney Int.*, **2003**, *63*, 1791.
[19] McMahon, E.G. *Curr. Opin. Pharmacol.*, **2001**, *1*, 190.
[20] Delyani, J.A. *Kidney Int.*, **2000**, *57*, 1408.
[21] Rocha, R.; Funder, J.W. *Ann. N. Y. Acad. Sci.*, **2002**, *970*, 89.
[22] Cella, J.; Brown, E.A.; Burtner, R.R. *J. Org. Chem.*, **1959**, *24*, 743.
[23] Cella, J.; Brown, E.A.; Burtner, R.R. *J. Org. Chem.*, **1959**, *24*, 1109.
[24] Selye, H. *Proc. Soc. Exp. Biol. Med.*, **1960**, *204*, 212.
[25] de Gasparo, M.; Joss, U.; Ramjoue, H.P.; Whitebread, S.E.; Haenni, H.; Schenkel, L.; Kraehenbuehl, C.; Biollaz, M.; Grob, J.; Schmidlin, J.; Wieland, P.; Wehrli, H.U. *J. Pharmacol. Exp. Ther.*, **1987**, *240*, 650.

[26] Takamura, N.; Maruyama, T.; Ahmed, S.; Suenaga, A.; Otagiri, M. *Pharm. Res.*, **1997**, *14*, 522.
[27] Quaschning, T.; Ruschitzka, F.; Shaw, S.; Luscher, T.F. *Hypertension*, **2001**, *37*, 801.
[28] Weinberger, M.H.; Roniker, B.; Krause, S.L.; Weiss, R.J. *Am. J. Hypertens.*, **2002**, *15*, 709.
[29] Weinberger, M.; MacDonald, T.; Conlin, P.R.; Roniker, B.; Patrick, J.L.; Krause, S. *Am. J. Hypertens.*, **2002**, *15*, A24.
[30] Burgess, E.; Niegowksa, J.; Tan, K.-W.; Kipnes, M.S.; Roniker, B.; Patrick, J.L.; Krause, S. *Am. J. Hypertens.*, **2002**, *15*, A23.
[31] Pitt, B.; Reichek, N.; Metscher, B.; Phillips, R.; Roniker, B.; Kleiman, J.; Burns, D. *Am. J. Hypertens.*, **2002**, *15*, A23.
[32] White, W.B.; Lewin, A.J.; Nestel, P.; Weiss, R.; Mansoor, G.A.; Roniker, B.; Kleiman, J.; Krause, S. *Am. J. Hypertens.*, **2002**, *15*, A23.
[33] Krum, H.; Nolly, H.; Workman, D.; He, W.; Roniker, B.; Krause, S.; Fakouhi, K. *Hypertension*, **2002**, *40*, 117.
[34] Delyani, J.A.; Robinson, E.L.; Rudolph, A.E. *Am. J. Physiol. Heart. Circ. Physiol.*, **2001**, *281*, H647.
[35] Suzuki, G.; Morita, H.; Mishima, T.; Sharov, V.G.; Todor, A.; Tanhehco, E.J.; Rudolph, A.E.; McMahon, E.G.; Goldstein, S.; Sabbah, H.N. *Circulation*, **2002**, *106*, 2967.
[36] Pitt, B.; Remme, W.; Zannad, F.; Neaton, J.; Martinez, F.; Roniker, B.; Bittman, R.; Hurley, S.; Kleiman, J.; Gatlin, M. *N. Engl. J. Med.*, **2003**, *348*, 1309.
[37] Epstein, M.; Buckalew, J.V.; Martinez, F.; Altamirano, J.; Roniker, B.; Kleiman, J.; Krause, S. *Am. J. Hypertens.*, **2002**, *15*, A24.
[38] Mangelsdorf, D.J.; Thummel, C.; Beato, M.; Herrlich, P.; Schutz, G.; Umesono, K.; Blumberg, B.; Kastner, P.; Mark, M.; Chambon, P.; Evans, R.M. *Cell*, **1995**, *83*, 835.
[39] Fuse, H.; Kitagawa, H.; Kato, S. *Mol. Endocrinol.*, **2000**, *14*, 889.
[40] Kitagawa, H.; Yanagisawa, J.; Fuse, H.; Ogawa, S.; Yogiashi, Y.; Okuno, A.; Nagasawa, H.; Nakajima, T.; Matsumoto, T.; Kato, S. *Mol. Cell Biol.*, **2002**, *22*, 3698.
[41] Rogerson, F.M.; Fuller, P.J. *Mol. Cell Endocrinol.*, **2003**, *200*, 45.
[42] Luisi, B.F.; Xu, W.X.; Otwinowski, Z.; Freedman, L.P.; Yamamoto, K.R.; Sigler, P.B. *Nature*, **1991**, *352*, 497.
[43] Rastinejad, F.; Perlmann, T.; Evans, R.M.; Sigler, P.B. *Nature*, **1995**, *375*, 203.
[44] Zhao, Q.; Khorasanizadeh, S.; Miyoshi, Y.; Lazar, M.A.; Rastinejad, F. *Mol. Cell*, **1998**, *1*, 849.
[45] Arriza, J.L.; Weinberger, C.; Cerelli, G.; Glaser, T.M.; Handelin, B.L.; Housman, D.E.; Evans, R.M. *Science*, **1987**, *237*, 268.
[46] Brzozowski, A.M.; Pike, A.C.; Dauter, Z.; Hubbard, R.E.; Bonn, T.; Engstrom, O.; Ohman, L.; Greene, G.L.; Gustafsson, J.A.; Carlquist, M. *Nature*, **1997**, *389*, 753.
[47] Shiau, A.K.; Barstad, D.; Loria, P.M.; Cheng, L.; Kushner, P.J.; Agard, D.A.; Greene, G.L. *Cell*, **1998**, *95*, 927.
[48] Tanenbaum, D.M.; Wang, Y.; Williams, S.P.; Sigler, P.B. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 5998.
[49] Matias, P.M.; Carrondo, M.A.; Coelho, R.; Thomaz, M.; Zhao, X.Y.; Wegg, A.; Crusius, K.; Egner, U.; Donner, P. *J. Med. Chem.*, **2002**, *45*, 1439.
[50] Bledsoe, R.K.; Montana, V.G.; Stanley, T.B.; Delves, C.J.; Apolito, C.J.; McKee, D.D.; Consler, T.G.; Parks, D.J.; Stewart, E.L.; Willson, T.M.; Lambert, M.H.; Moore, J.T.; Pearce, K.H.; Xu, H.E. *Cell*, **2002**, *110*, 93.
[51] Kauppi, B.; Jakob, C.; Farnegardh, M.; Yang, J.; Ahola, H.; Alarcon, M.; Calles, K.; Engstrom, O.; Harlan, J.; Muchmore, S.; Ramqvist, A.K.; Thorell, S.; Ohman, L.; Greer, J.; Gustafsson, J.A.; Carlstedt-Duke, J.; Carlquist, M. *J. Biol. Chem.*, **2003**, *278*, 22748.
[52] Fagart, J.; Wurtz, J.M.; Souque, A.; Hellal-Levy, C.; Moras, D.; Rafestin-Oblin, M.E. *EMBO J.*, **1998**, *17*, 3317.
[53] Geller, D.S.; Farhi, A.; Pinkerton, N.; Fradley, M.; Moritz, M.; Spitzer, A.; Meinke, G.; Tsai, F.T.; Sigler, P.B.; Lifton, R.P. *Science*, **2000**, *289*, 119.
[54] Liu, W.; Wang, J.; Sauter, N.K.; Pearce, D. *Proc. Natl. Acad. Sci. USA*, **1995**, *92*, 12480.
[55] Trapp, T.; Holsboer, F. *Trends Pharmacol. Sci.*, **1996**, *17*, 145.
[56] Bamberger, C.M.; Bamberger, A.M.; Wald, M.; Chrousos, G.P.; Schulte, H.M. *J. Steroid. Biochem. Mol. Biol.*, **1997**, *60*, 43.
[57] Westin, S.; Rosenfeld, M.G.; Glass, C.K. *Adv. Pharmacol.*, **2000**, *47*, 89.
[58] Heery, D.M.; Kalkhoven, E.; Hoare, S.; Parker, M.G. *Nature*, **1997**, *387*, 733.
[59] Darimont, B.D.; Wagner, R.L.; Apriletti, J.W.; Stallcup, M.R.; Kushner, P.J.; Baxter, J.D.; Fletterick, R.J.; Yamamoto, K.R. *Genes. Dev.*, **1998**, *12*, 3343.
[60] Leo, C.; Chen, J.D. *Gene*, **2000**, *245*, 1.
[61] Ding, X.F.; Anderson, C.M.; Ma, H.; Hong, H.; Uht, R.M.; Kushner, P.J.; Stallcup, M.R. *Mol. Endocrinol.*, **1998**, *12*, 302.
[62] Hong, H.; Kohli, K.; Garabedian, M.J.; Stallcup, M.R. *Mol. Cell Biol.*, **1997**, *17*, 2735.
[63] Torchia, J.; Rose, D.W.; Inostroza, J.; Kamei, Y.; Westin, S.; Glass, C.K.; Rosenfeld, M.G. *Nature*, **1997**, *387*, 677.
[64] Lowell, B.B.; Spiegelman, B.M. *Nature*, **2000**, *404*, 652.
[65] Knutti, D.; Kralli, A. *Trends Endocrinol. Metab.*, **2001**, *12*, 360.
[66] Shang, Y.; Hu, X.; DiRenzo, J.; Lazar, M.A.; Brown, M. *Cell*, **2000**, *103*, 843.
[67] Ordentlich, P.; Downes, M.; Evans, R.M. *Curr. Top. Microbiol. Immunol.*, **2001**, *254*, 101.
[68] Hu, X.; Lazar, M.A. *Nature*, **1999**, *402*, 93.
[69] Germain, P.; Iyer, J.; Zechel, C.; Gronemeyer, H. *Nature*, **2002**, *415*, 187.
[70] Fejes-Toth, G.; Pearce, D.; Naray-Fejes-Toth, A. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 2973.
[71] Couette, B.; Fagart, J.; Jalaguier, S.; Lombes, M.; Souque, A.; Rafestin-Oblin, M.E. *Biochem. J.*, **1996**, *315* (Pt. 2), 421.
[72] Bruner, K.L.; Derfoul, A.; Robertson, N.M.; Guerriero, G.; Fernandes-Alnemri, T.; Alnemri, E.S.; Litwack, G. *Recept. Signal. Transduct.*, **1997**, *7*, 85.
[73] Bamberger, C.M.; Wald, M.; Bamberger, A.M.; Schulte, H.M. *Mol. Cell Endocrinol.*, **1997**, *131*, 233.
[74] Webster, M.K.; Goya, L.; Ge, Y.; Maiyar, A.C.; Firestone, G.L. *Mol. Cell Biol.*, **1993**, *13*, 2031.
[75] Mick, V.E.; Itani, O.A.; Loftus, R.W.; Husted, R.F.; Schmidt, T.J.; Thomas, C.P. *Mol. Endocrinol.*, **2001**, *15*, 575.
[76] Derfoul, A.; Robertson, N.M.; Lingrel, J.B.; Hall, D.J.; Litwack, G. *J. Biol. Chem.*, **1998**, *273*, 20702.
[77] Kolla, V.; Robertson, N.M.; Litwack, G. *Biochem. Biophys. Res. Commun.*, **1999**, *266*, 5.
[78] Brennan, F.E.; Fuller, P.J. *Mol. Cell Endocrinol.*, **2000**, *166*, 129.
[79] Shigaev, A.; Asher, C.; Latter, H.; Garty, H.; Reuveny, E. *Am. J. Physiol. Renal. Physiol.*, **2000**, *278*, F613.
[80] Naray-Fejes-Toth, A.; Fejes-Toth, G. *Kidney Int.*, **2000**, *57*, 1290.
[81] Chen, S.Y.; Bhargava, A.; Mastroberardino, L.; Meijer, O.C.; Wang, J.; Buse, P.; Firestone, G.L.; Verrey, F.; Pearce, D. *Proc. Natl. Acad. Sci. USA*, **1999**, *96*, 2514.
[82] Staub, O.; Dho, S.; Henry, P.; Correa, J.; Ishikawa, T.; McGlade, J.; Rotin, D. *EMBO J.*, **1996**, *15*, 2371.
[83] Staub, O.; Gautschi, I.; Ishikawa, T.; Breitschopf, K.; Ciechanover, A.; Schild, L.; Rotin, D. *EMBO J.*, **1997**, *16*, 6325.
[84] Snyder, P.M.; Olson, D.R.; Thomas, B.C. *J. Biol. Chem.*, **2002**, *277*, 5.
[85] Shimkets, R.A.; Warnock, D.G.; Bositis, C.M.; Nelson-Williams, C.; Hansson, J.H.; Schambelan, M.; Gill, J.R. Jr.; Ulick, S.; Milora, R.V.; Findling, J.W.; Canessa, C.M.; Rossier, B.C.; Lifton, R.P. *Cell*, **1994**, *79*, 407.
[86] Hansson, J.H.; Nelson-Williams, C.; Suzuki, H.; Schild, L.; Shimkets, R.; Lu, Y.; Canessa, C.; Iwasaki, T.; Rossier, B.; Lifton, R.P. *Nat. Genet.*, **1995**, *11*, 76.
[87] Shimkets, R.A.; Lifton, R.P.; Canessa, C.M. *J. Biol. Chem.*, **1997**, *272*, 25537.
[88] Chang, S.S.; Grunder, S.; Hanukoglu, A.; Rosler, A.; Mathew, P.M.; Hanukoglu, I.; Schild, L.; Lu, Y.; Shimkets, R.A.; Nelson-Williams, C.; Rossier, B.C.; Lifton, R.P. *Nat. Genet.*, **1996**, *12*, 248.
[89] Strautnieks, S.S.; Thompson, R.J.; Gardiner, R.M.; Chung, E. *Nat. Genet.*, **1996**, *13*, 248.
[90] Feraille, E.; Doucet, A. *Physiol. Rev.*, **2001**, *81*, 345.
[91] Todd-Turla, K.M.; Schnermann, J.; Fejes-Toth, G.; Naray-Fejes-Toth, A.; Smart, A.; Killen, P.D.; Briggs, J.P. *Am. J. Physiol.*, **1993**, *264*, F781.
[92] Seckl, J.R.; Dickson, K.L.; Yates, C.; Fink, G. *Brain Res.*, **1991**, *561*, 332.
[93] Kornel, L.; Nelson, W.A.; Manisundaram, B.; Chigurupati, R.; Hayashi, T. *Steroids*, **1993**, *58*, 580.
[94] Funder, J.W. *Kidney Int.*, **2000**, *57*, 1358.
[95] Mune, T.; Rogerson, F.M.; Nikkila, H.; Agarwal, A.K.; White, P.C. *Nat. Genet.*, **1995**, *10*, 394.
[96] Funder, J.W.; Pearce, P.T.; Smith, R.; Smith, A.I. *Science*, **1988**, *242*, 583.

[97] Stewart, P.M.; Corrie, J.E.; Shackleton, C.H.; Edwards, C.R. *J. Clin. Invest.*, **1988**, *82*, 340.
[98] Funder, J.W.; Pearce, P.T.; Myles, K.; Roy, L.P. *FASEB J.*, **1990**, *4*, 3234.
[99] Young, M.; Fullerton, M.; Dilley, R.; Funder, J. *J. Clin. Invest.*, **1994**, *93*, 2578.
[100] Qin, W.; Rudolph, A.E.; Bond, B.R.; Rocha, R.; Blomme, E.A.; Goellner, J.J.; Funder, J.; McMahon, E. *Circ. Res.*, **2003**, In press.
[101] Robson, A.C.; Leckie, C.M.; Seckl, J.R.; Holmes, M.C. *Brain. Res. Mol. Brain. Res.*, **1998**, *61*, 1.
[102] Young, M.J.; Moussa, L.; Dilley, R.; Funder, J.W. *Endocrinology*, **2003**, *144*, 1121.
[103] Rogerson, F.M.; Dimopoulos, N.; Sluka, P.; Chu, S.; Curtis, A.J.; Fuller, P.J. *J. Biol. Chem.*, **1999**, *274*, 36305.
[104] Thiemermann, C. *Nat. Med.*, **2002**, *8*, 453.
[105] Fiebeler, A.; Schmidt, F.; Muller, D.N.; Park, J.K.; Dechend, R.; Bieringer, M.; Shagdarsuren, E.; Breu, V.; Haller, H.; Luft, F.C. *Hypertension*, **2001**, *37*, 787.
[106] Jeunemaitre, X.; Chatellier, G.; Kreft-Jais, C.; Charru, A.; DeVries, C.; Plouin, P.F.; Corvol, P.; Menard, J. *Am. J. Cardiol*., **1987**, *60*, 820.
[107] de Gasparo, M.; Whitebread, S.E.; Preiswerk, G.; Jeunemaitre, X.; Corvol, P.; Menard, J. *J. Steroid. Biochem.*, **1989**, *32*, 223.
[108] Whitebread, S.E.; Haenni, H.; Ramjoue, H.P.; Joss, U.; Kraehenbuehl, C.; Schenkel, L. *Develop. Nephrol.*, **1987**, *18*, 550.