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The regulation of cell growth and survival by aldosterone

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1. ABSTRACT

The steroid hormone aldosterone is synthesized from cholesterol, mainly in the *zona glomerulosa* of the adrenal cortex. Aldosterone exerts its effects in the epithelial tissues of the kidney and colon and in non-epithelial tissues such as the brain and cardiovasculature. The genomic response to aldosterone involves dimerization of the mineralocorticoid receptor (MR), dissociation of heat shock proteins from MR, translocation of the aldosterone-MR complex to the nucleus and the concomitant regulation of gene expression. Rapid responses to aldosterone occur within seconds to minutes, do not involve transcription or translation and can modulate directly or indirectly the later genomic responses. Aside from the well-known effects of aldosterone on the regulation of sodium and water homeostasis, aldosterone can also produce deleterious structural changes in tissues by inducing hypertrophy and the dysregulation of proliferation and apoptosis, leading to fibrosis and tissue remodelling. Here we discuss the involvement of aldosterone-mediated rapid signalling cascades in the development of disease states such as chronic kidney disease and heart failure, and the antagonists that can inhibit these pathophysiological responses.

2. INTRODUCTION

2.1. The mineralocorticoid receptor

The mineralocorticoid receptor (MR) is a member of the steroid receptor superfamily. All members of this family can be subdivided into three principal structural domains, consisting of an NH_2-terminal domain of variable length and sequence (<15% amino acid identity between receptors); a highly conserved DNA binding domain (> 40% identity between receptors); and a COOH-terminal ligand-binding domain, which is highly variable in amino acid sequence (<15%-57% amino acid identity across the family) (1). Within this receptor family, MR and the glucocorticoid receptor (GR) are characterized by relatively high amino acid identity in the ligand binding domain (57%) and very high homology in the DNA-binding domain (94%). Aldosterone binds to MR with strong affinity whereas binding to GR occurs with much lower affinity. Glucocorticoids (cortisol in humans and corticosterone in rats and mice) bind both the mineralocorticoid receptor and glucocorticoid receptor with strong affinity. However, although the *in vitro* K_d values for binding of aldosterone and glucocorticoids to MR are similar, the binding kinetics are not identical, as the K_{off} rate for glucocorticoids is approximately 5 times faster than

that of aldosterone (2). Upon activation, both the aldosterone-MR and glucocorticoid-GR ligand-receptor complexes bind to a consensus DNA sequence, the glucocorticoid responsive element (GRE) (3). The existence of tissue-specific proteins that modulate the response of the GRE according to bound hormone (aldosterone or cortisol) has been postulated. For example, the human elongation factor ELL (eleven-nineteen lysine-rich leukemia) is a coactivator of MR, increasing MR-mediated transactivation by 2.5 fold and at the same time acting as a co-repressor for GR, inhibiting transactivation by this receptor by 90% (4).

2.2. 11-beta-HSD2-mediated specificity of aldosterone for MR

MR has equivalent high affinity for binding the mineralocorticoid aldosterone and the glucocorticoids cortisol and corticosterone (5). In the classical aldosterone target tissues such as kidney and colon, the specificity of aldosterone for MR is maintained by the enzyme hydroxysteroid dehydrogenase type 2 (11-beta-HSD2) which reduces the abundant cortisol/corticosterone to inactive 11-keto metabolites, thus allowing preferential access for aldosterone to MR (6, 7). There exists a ~1000-fold higher level of circulating total glucocorticoids and a ~100-fold level of plasma free glucocorticoids *in vivo*. Therefore, in other target tissues of aldosterone such as the heart and parts of the brain, where there is little or no expression of 11-beta-HSD2, MR will be predominantly occupied by glucocorticoids (8). In patients with the rare hypertensive syndrome of apparent mineralocorticoid excess (AME) in which there is an absence of 11-beta-HSD2, or when the enzyme is inhibited by glycyrrhizic acid in liquorice (9), glucocorticoids can occupy and activate renal MR (8). Moreover, it has been suggested that glucocorticoids may activate MR when the intracellular redox state changes, as NAD is a cosubstrate in the generation of cortisone from cortisol (10, 11).

Cortisol is less efficient than aldosterone in stimulating MR transactivation in an *in vitro* system (12); however, *in vivo*, any differences in efficiency of transactivation by aldosterone, cortisol or corticosterone are clinically insignificant compared to the abundance of circulating glucocorticoids over mineralocorticoids, unless there is a mechanism to increase local concentrations of aldosterone. Extra-renal sites of aldosterone production have been identified, including brain (13), blood vessels (14) and heart (15, 16). In the vasculature, aldosterone production has been reported in both endothelial and smooth muscle cells (17, 18). Local synthesis of aldosterone in close proximity to cells expressing MR, could give aldosterone a MR-binding advantage over glucocorticoids in the absence of 11-beta-HSD2, but the physiological relevance of extra-renal aldosterone production is to date unknown.

2.3. MR antagonists as therapeutic agents

Fifty years ago, Selye had already described myocardial necrosis and fibrosis in animal models with elevated aldosterone levels and how the aldosterone receptor blocker, spironolactone, could attenuate these effects (19). More recently, in animal models of hypertension, such as stroke-prone spontaneously hypertensive rats, aldosterone receptor antagonism reduced vascular remodelling and renal damage, particularly in a high salt diet condition (20-23). Furthermore, in a rat model of heart disease, spironolactone significantly reduced fibrosis and atrial excitability (24). Eplerenone, another MR antagonist, is similar in structure to spironolactone but has greater mineralocorticoid specificity – and consequently fewer anti-androgenic and progesterone-like effects. Eplerenone protected the myocardium and coronary vasculature in rat models of aldosterone-mediated disease (25, 26). In aldosterone-infused rats, spironolactone or eplerenone prevented inflammation and fibrosis in the heart, blood vessels and kidney, improved endothelial function, and reduced activation of NAD (P)H oxidase (27-31). Importantly, both spironolactone and eplerenone significantly ameliorate severe heart failure at concentrations that do not lower blood pressure (32-34).

3. ALDOSTERONE AND RENAL FUNCTION

The kidney is a key target for the renin-angiotensin-aldosterone system (RAAS). The action of the RAAS cascade modulates systemic blood pressure through the regulation of electrolyte transport. A key facet of RAAS is the regulation of Na^+ re-absorption from the renal ultra-filtrate in the distal nephron through the modulation of the activity of the epithelial Na^+ channel (ENaC) and Na^+/K^+ pump. Dysfunctional RAAS cascade activation, resulting from congenital or acquired defects contribute to hypertension (35), which is a significant risk factor in the development of cardiovascular disease. The individual receptors (angiotensin receptor, mineralocorticoid receptor), enzymes (angiotensin converting enzyme- ACE) and effectors (ENaC) of the RAAS cascade are important therapeutic targets in the treatment of hypertension. Aldosterone exerts its effects in tissues that express MR (36). In the kidney these include the distal nephron and the mesangial cells located in the juxtaglomerular apparatus (37). The action of aldosterone on the epithelial cells of the distal nephron modulates the activity of ion transporters that regulate electrolyte secretion and re-absorption and acid-base balance. Aldosterone regulates transporter activity through genomic actions via MR through the modulation of transporter subunit and regulatory protein expression. Aldosterone also causes the rapid non-genomic activation of signalling cascades that modulate transporter activity directly, or by impacting upon the processes of sub-cellular trafficking and degradation of transporter proteins. The synergism between the rapid and genomic effects of aldosterone in epithelia of the kidney, colon and sweat gland leads to the precise modulation of ion fluxes that impact upon systemic electrolyte balance.

The homeostatic regulation of Na^+ re-absorption and K^+ secretion are held to be the most important physiological functions of aldosterone in the kidney. However, the growth effects of aldosterone on the MR-expressing cells of the nephron are more complex, and evidence points to aldosterone promoting the differentiation of renal cells in nephron development as well as

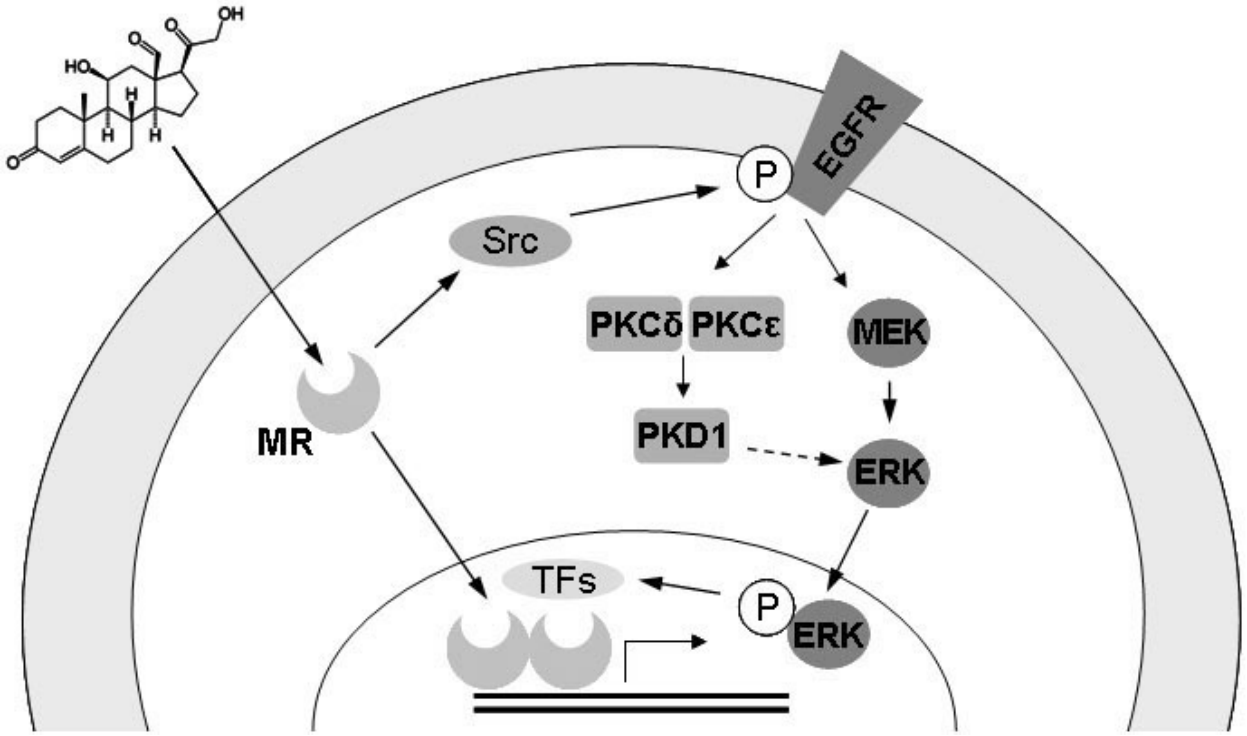

Figure 1. Aldosterone-mediated rapid extra-nuclear signalling pathways and latent genomic responses in the cortical collecting duct. Aldosterone binds to the mineralocorticoid receptor, inducing dimerization and translocation to the nucleus where the hormone-receptor complex acts as a ligand-dependent transcription factor regulating the expression of genes. Aldosterone also activates c-Src which phosphorylates EGFR and leads to its trans-activation. Activated EGFR rapidly activates both a PKC-PKD1 signaling pathway and a MEK-ERK pathway, both of which are closely inter-related. Phosphorylated ERK in the nucleus can act on transcriptional factors modulating the transcriptional response. These rapidly-induced signaling pathways are essential in transducing the aldosterone-mediated proliferation of renal cortical collecting duct cells (45). (PKCdelta protein kinase C delta, PKCepsilon protein kinase C epsilon, PKD1 protein kinase D1, EGFR epidermal growth factor receptor, MEK mitogen-activated protein kinase, ERK extracellular signal-regulated kinase).

contributing to the pathology of renal diseases through dysregulation of cell growth. The deleterious effects of circulating aldosterone on the histology of the kidney have been attributed to systemic hypertension; however, the direct effects of aldosterone on MR-expressing cells in the nephron may also contribute to renal damage. Recent evidence points to the modulation of renal cell growth by aldosterone both in culture and *in vivo*. Aldosterone stimulates the proliferation and differentiation of renal stem cells to produce pseudo-tubules in culture (38), supporting data obtained in animal models (39). Dysregulation of these developmental processes can contribute to the pathology of kidney diseases through the promotion of renal fibrosis or hyperplasia of the epithelial cells in the MR-expressing segments of the nephron. In common with the effects of aldosterone on renal electrolyte transport, the growth stimulatory effects of aldosterone also result from a synergism between the latent transcriptional activity of MR and the protein kinase signalling cascades rapidly stimulated by the hormone (Figure 1).

3.1. Aldosterone and nephron differentiation

The nephron is the functional unit of the kidney and each nephron arises from paired progenitor cells that act in synchrony through proliferation and differentiation to produce a complete tubule. The nephrogenic mesenchymal stem cells develop into most of the segments of the nephron including the proximal tubule, the loop of Henle and the distal tubule, while a different sub-set of stem cells found at the collecting duct ampullae form the collecting duct tubule itself. The earliest distinguishable stage in nephron development is marked by mesenchymal stem cell proliferation, which results in the development of S-shaped tubule bodies. The molecular mechanisms underpinning the synergism between the two stem cell sub-sets has not yet been established. Renal stem cells isolated from neonatal animals can be cultured *in vitro* on porous matrices (40). Experiments to identify growth factors required to promote differentiation of these stem cells identified the inclusion of aldosterone as a prerequisite for renal stem cell differentiation (38, 41). This effect could not be mimicked by EGF or other steroid hormones. The structures that develop show some degree of cellular differentiation with the modification of surface glycoproteins by the addition of the collecting duct-specific marker *N*-acetylgalactosamine detectable, and the establishment of tight junctions. These effects of aldosterone have not been detected using cells of the renal tubule isolated from adult animals.

Table 1. Aldosterone-mediated signalling events leading to cell proliferation (P), hypertrophy (H), apoptosis (A) or survival (S)

Aldosterone	Signalling Event	Time-scale	Blocker	Cell Type	Outcome	Ref.
10nM	ERK1/2 phosphorylation	2 min	10μM RU28318	[1]M1-CCD	P	(45)
1nM	PKCalpha activation/ translocation	5-15 min	-	[2]$RCCD_2$	-	(42)
1-10nM/L	ERK1/2 phosphorylation	10 min	10μM eplerenone	rat mesangial	P	(37)
10^{-6}M	Bad dephosphorylation	16 h	10^{-5}M spironolactone	human mesangial	A	(48)
1.5μM	MAPK	5 min	RU486 (GR)	Renal A6	-	(44)
10nM	Phospho-Raf, Phospho-MEK Phospho-MAPK	2 h	0.1μM spironolactone	Rat cardiac fibroblasts	P	(148)
25nM/L	PKD1 phosphorylation	30 min	100nM/L spironolactone	Neonatal rat cardiac myocytes	H	(103)
5μmol/L	Phospho-ERK1/2 Phospho-JNK PKC translocation	5 min	10μM/L spironolactone 1μM/L mifepristone	Neonatal rat cardiac myocytes	H	104)
10^{-6}M	NHE activity increased	3 hrs	-	Rat VSMCs	-	(115)
10^{-9}mol/L to 10^{-7}mol/L	NADPH oxidase activation, [3]ASK1 phosphorylation	5 min	eplerenone	Neonatal rat cardiac myocytes	A	(127)
10 pmol/L	phospho-ERK1/2, phospho-p70 S6K	-	-	[4]BAEC	-	(137)
0.1 μmol/L	c-Src phosphorylation p38MAPK	1-5min 1 min	10μmol/L eplerenone	Rat VSMCs	-	(143)
10nM	BMK phosphorylation	10 min	10 μmol/L eplerenone	[5]RASMCs	P	145)
Dex 10^{-5}M Aldo 10^{-5}M	- -	48h 48h	- -	Rat hippocampal primary culture	A S	(194)

[1]M1-CCD, murine cortical collecting duct cell line, [2]$RCCD_2$, rat cortical collecting duct cell line, [3]ASK1, Apoptosis signal-regulating kinase 1, [4]BAEC, Bovine aortic endothelial cell, [5]RASMC, Rat aortic smooth muscle cells

The mechanism by which aldosterone promotes renal cell growth is unclear but evidence points to the involvement of protein kinase signalling cascades that modulate the transcriptional effects of MR. Multiple signalling intermediates which promote cell growth in response to other stimuli are activated by aldosterone in the distal nephron; these include PKCalpha (42), PKD1 (43) and ERK1/2 mitogen activated protein (MAP) kinase (44) (Table 1). The activation of ERK1/2 in response to aldosterone occurs in two phases; an initial transient phase within 5 min and a second sustained phase after 30 min (45). The sustained phase of ERK1/2 activation is dependent on the concurrent activation of protein kinase D. Similar stabilization of ERK1/2 activation is also observed when ERK1/2 is activated in fibroblasts in response to GPCR agonists, and this sustained activation of ERK1/2 is necessary to promote cell cycle progression (46). The transient activation of ERK1/2 by aldosterone is not adequate to stimulate cell proliferation.

The importance of aldosterone and ERK1/2 in renal development is confirmed *in vivo*. When new born rats were treated for 7 days with the MR antagonist spironolactone, there was an increase in the abundance of apoptotic cells observed in renal histology sections (47). These apoptotic cells were predominantly detected in the segments of the nephron where MR is most highly expressed and where aldosterone exerts its most pronounced physiological effects. Spironolactone treatment caused suppression of ERK1/2 and p38 MAP kinase protein expression; however, there was an increase in the abundance of the mRNA of both kinases. Spironolactone treatment induced the expression of JNK-2 in the glomeruli and cortical tubules (47). These data from newborn tissues are also reflected to some extent in data from mature tissues. Aldosterone treatment stimulates both apoptosis and proliferation in human mesangial cells, and correlates with the de-phosphorylation of Bad, a Bcl-2 family protein and with the release of cytochrome c from mitochondria (48). Aldosterone treatment of mesangial cells also results in ERK1/2 activation within 10 min that can promote proliferation and this effect is blocked by the MR antagonist eplerenone (37). MR is abundantly expressed by mesangial cells, and is predominantly localized to the cell cytoplasm with only a small fraction in the nucleus and barely detectable quantities associated with the cell membrane (37). Aldosterone was also pro-apoptotic *in vivo*; systemic treatment resulted in rat mesangial cells becoming apoptotic, an effect that was eplerenone-sensitive and correlated with an increase in systolic blood pressure and albuminuria (48). It appears that the effects of aldosterone on apoptosis and proliferation are dependent on the stage of tissue development; with different growth effects on mature as compared to immature renal tissues.

3.2. Aldosterone and nephropathy

The dysregulation of cell growth contributes to the development and progression of renal pathologies including chronic kidney disease, polycystic kidney disease and diabetes-associated nephropathy. The stimulation of cell proliferation in the nephron by aldosterone may be a significant factor that contributes to the rate of progression of these chronic conditions. The deterioration in renal function results from aberrant cell proliferation and apoptosis, and from the inappropriate deposition of extracellular matrix material that results in renal fibrosis and localized inflammation. Renal damage associated with diabetic and non-diabetic nephropathies can be ameliorated by MR antagonism, an effect that is further enhanced by concurrent RAAS blockade, using angiotensin-converting enzyme (ACE) inhibitors (49). Experiments using a diabetic rat model showed that MR antagonism with eplerenone employed in combination with enalapril an ACE inhibitor, enhanced the glomerular filtration rate and helped control glomerulosclerosis with a simultaneous decline in the expression of TGF-β1, Collagen type-IV and plasminogen activator factor-1 (50). The production of reactive oxygen species (ROS) has been detected in the renal tissues of rats infused with aldosterone, and aldosterone-induced renal damage correlates with

molecular markers for ROS production. Aldosterone promotes ROS release through increased NADPH oxidase activity, which is associated with transcriptional up-regulation and membrane association of NADPH oxidase subunits in renal cells, ROS production contributes to renal fibrosis through the promotion of apoptosis, and this ROS response to aldosterone can be suppressed by eplerenone treatment and also mitigated by the administration of antioxidants (51).

The NF-kappaB/ RelA family of transcription factors are key mediators of apoptosis and inflammation. The activation of NF-kappaB-dependent transcription is characteristic of inflammatory and fibrotic responses in MR-expressing tissues such as the liver, heart and kidney. NF-kappaB activation in the principal cells of the collecting duct is up-regulated in response to aldosterone treatment; this promotion of fibrosis mediated by the transcription-dependent stimulation of SGK1 activity by aldosterone rather than through the stimulation of proliferation-linked MAP kinases that has been proposed as an alternative mechanism. The expression of key pro-inflammatory cytokines including IL-1β and IL-6 is promoted by NF-kappaB-dependent transcriptional regulation, and so promotes a localized inflammatory response (52). The stimulation of NF-kappaB-dependent transcription in the collecting duct principal cells is MR-dependent and can in fact be suppressed by simultaneous stimulation of the glucocorticoid receptor using dexamethasone. Cellular fibrosis is characterised by a marked shift in the structure of the actin cytoskeleton. Mesangial cells treated with aldosterone display an increase in the phosphorylation state of myosin phosphatase target subunit-1 within twenty minutes of treatment. This protein is a substrate for Rho kinase (ROK) and there is an increase in actin polymerization detectable over the same time frame of hormone treatment (53). ROK is a key regulator of actin structure and subcellular trafficking. In consequence, aldosterone has the capacity to initiate extensive cytoskeletal remodelling in the mesangial cells through the ROK cascade. The stimulation of cell metabolism, or hypertrophy by aldosterone treatment in mesangial cells correlates with a stimulation in the expression of collagen types I, III and IV and also of smooth muscle actin-α protein, a molecular marker for myofibroblastic transformation of mesangial cells. These aldosterone-induced changes in cytoskeletal organization and gene expression could be blocked by MR antagonism and also by the ROK inhibitor Y27632; so demonstrating the involvement of this signalling pathway in the effects of aldosterone on mesangial cell differentiation and growth associated with fibrosis.

3.3. Aldosterone and polycystic kidney disease

The unregulated proliferation of cells in the epithelium of the distal renal tubule is a contributory factor in the progression of polycystic kidney disease (54). Genetic mutations in the chromosomal genes encoding fibrocystin or polycystin 1 and 2, confers susceptibility in developing autosomal recessive polycystic kidney disease (ARPKD) and autosomal dominant polycystic kidney disease (ADPKD) respectively (55, 56). The proteins encoded by these genes are multi-functional structural membrane proteins that have roles in epithelial polarization, tight junction assembly, Ca^{2+} transport and intracellular signalling. Subjects who experience chronic hypertension have accelerated progression of polycystic kidney disease (57) and therapeutic control of the hypertension through MR antagonism is one strategy for intervention (58). The development of fluid filled renal cysts and progressive cyst enlargement is a result of epithelial cell proliferation (59) and the dysregulation of trans-epithelial ion transport. The initial formation of cysts in ARPKD is largely restricted to the epithelium in segments of the distal nephron that express MR (59); a potential role for MR and aldosterone action in stimulating aberrant cell proliferation deserves further investigation.

Fibrocystin gene silencing in the HEK293 renal cell line resulted in cells that displayed a hyper-proliferative phenotype following EGF treatment and also resulted in amplified activation of the ERK1/2 signalling cascade in response to EGF (60). Heparin-bound EGF is over-expressed in ARPKD and antibodies antagonistic to EGFR suppressed the mitogenicity of cleared cystic fluid (61). EGFR expression by the renal tubule is sensitive to aldosterone (62) and is required to transduce the activation of rapid signalling responses by aldosterone in the CHO cell line (63) and in renal collecting duct cells (45).

The stimulation of the ERK1/2 signalling cascade by aldosterone in the cells of the distal nephron is coupled to EGFR trans-activation (64-66), and the nuclear localization of ERK1/2 occurs subsequent to its activation by aldosterone and is consistent with its role as a regulator of transcription factors. Furthermore, attenuation of sustained ERK1/2 activation through PKD1 suppression antagonises the nuclear stabilization of MR in response to aldosterone (45). The nuclear translocation of ERK1/2 in response to the aldosterone treatment of murine cortical collecting duct cells (M1-CCD) was blocked by EGFR inhibition; consequently there is a coupling of EGFR signalling to MR nuclear association and transcription through the activation of the ERK1/2 signalling cascade (67). Therapeutic intervention in ADPKD through antagonism of the RAAS to stabilize hypertension in order to slow cyst enlargement is the subject of an on-going clinical investigation (68). Antagonism of aldosterone action may also have a more direct effect on ADPKD progression by suppressing proliferation in the epithelial cells making up the renal cysts and so helping to control renal damage associated with this disease.

4. ALDOSTERONE AND THE CARDIOVASCULAR SYSTEM

Chronic heart failure is a multi-factorial syndrome. Aldosterone mediates a wide range of effects, through both rapid signalling events and long-term genomic responses, in each cell type making up the cardiovascular system, including cardiac fibroblasts, cardiomyocytes, vascular smooth muscle cells (VSMCs) and endothelial cells. Dysregulation of aldosterone-sensitive physiological processes in each of these cell types can lead to pathological consequences.

The activity of RAAS is significantly increased during hypertrophy and heart failure. This results not only in changes in blood pressure and volume but also in direct deleterious effects on cardiac and vascular structure. Prolonged stimulation of RAAS becomes maladaptive leading to excessive vasoconstriction, fibrosis and cardiac remodelling (69). Aldosterone-mediated activation of MR can have direct effects on the myocardium and vasculature, leading to detrimental processes such as hypertrophy, necrosis, fibrosis and endothelial dysfunction, all of which contribute to the pathophysiology of heart failure. The direct adverse effects of aldosterone can lead to an increased risk of arrhythmic death (70, 71). It has been shown that aldosterone rapidly increased free $(Ca^{2+})_i$ in vascular smooth muscle and endothelial cells (72, 73) and many studies have outlined the modulation of calcium influx as a central factor in the pathophysiological action of aldosterone in the heart (74-78).

Pharmacological intervention with MR antagonists has clearly demonstrated the importance of aldosterone in mediating cardiac fibrosis in humans and in animal models of heart failure. The role aldosterone plays in cardiovascular damage in humans was highlighted by the randomized Aldactone evaluation study (RALES) (33) and the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) (32) trials, which showed that addition of an MR antagonist to standard therapy in heart failure patients reduces cardiac morbidity and mortality (33). These MR antagonists significantly ameliorated severe heart failure at concentrations that do not lower blood pressure (32-34). Furthermore, another clinical trial, the "4E Study," demonstrated that hypertensive patients treated with the aldosterone antagonist eplerenone alone (without an ACE inhibitor) showed marked regression of left ventricular (LV) hypertrophy, a marker for cardiovascular disease (79).

4.1. Aldosterone and cardiac dysfunction

The proliferation of cardiac fibroblasts and excess matrix deposition are two key factors that contribute to the development of cardiac hypertrophy and heart failure. Experimental (80-84) and clinical (79, 85, 86) studies have suggested that aldosterone, and the blockade of MR with the antagonists spironolactone or eplerenone, modulate LV hypertrophy. Aldosterone induced both hypertrophic and fibrotic responses in the heart tissue of uninephrectomized rats under conditions of excessive salt ingestion (80-82). Moreover, aldosterone stimulated collagen synthesis in cultured rat cardiac fibroblasts and a direct effect was found for aldosterone exposure on cardiac fibroblasts in mediating myocardial fibrosis in hypertensive heart disease (87).

Cardiomyocytes do not normally express 11-beta-HSD2 and therefore cardiac MR is normally occupied by physiological glucocorticoids. After tissue damage, ROS generation and changes in intracellular redox status could lead to the activation of glucocorticoid-occupied MR, as suggested by preliminary studies on isolated cardiomyocytes (88). On the other hand, the cardiomyocyte-selective overexpression of 11-beta-HSD2 in mice, which allowed aldosterone to access cardiomyocyte MR *in vivo*, was followed by cardiac hypertrophy and fibrosis (89).

Local production of aldosterone can be considered a means to counteract lack of 11-beta-HSD2 expression. Locally produced aldosterone may play a role in promoting vascular and myocardial fibrosis, independent of its effects on hemodynamic regulation and fluid homeostasis (90, 91). The production of aldosterone was shown to be increased in the failing human ventricle in proportion to heart failure severity (92) and a positive correlation was found between circulating aldosterone levels and increased left ventricular mass in non-selected populations (93) as well as in patients with essential hypertension (85). An Aldosterone Synthase Inhibitor (ASI), FAD286, is effective in reducing cardiac damage, even in the presence of normal glucocorticoid levels (94, 95). This provides evidence for aldosterone and not glucocorticoids to be the major pathophysiological agonist at MR in cardiovascular tissues. However, species-specific differences exist, for example, aldosterone synthase mRNA is expressed in some strains of rat, but not in mouse heart, and although Wistar and spontaneously hypertensive (SHR) rats express aldosterone synthase mRNA in cardiac tissue under basal conditions, aldosterone synthase expression is only observed in Sprague Dawley rat hearts after chronic treatment with angiotensin II (96). Moreover, most of the aldosterone in the heart tissue of healthy rats was shown to be derived from the circulation and the amount of aldosterone synthesized in the heart was found to be minimal (97).

4.2. Rapid aldosterone signalling in cardiac myocytes

Aldosterone induces the expression of a number of genes in cardiac myocytes, including the Na^+/K^+-ATPase (98), angiotensin converting enzyme (ACE) (99), angiotensin type 1 receptor (AT_1R) (100, 101) and cardiotrophin-1 (CT-1), a cytokine involved in the induction of hypertrophy in both neonatal and adult cardiomyocytes (102). Apart from these transcriptional effects, aldosterone also mediates non-genomic rapid signalling events that lead to cardiac myocyte hypertrophy and the aberrant regulation of proliferation and apoptosis (Table 1), all of which eventually lead to pathophysiological disease states. Aldosterone mediates deleterious effects in cultured neonatal rat cardiac myocytes by promoting hypertrophy. This was shown to occur through a protein kinase D1-dependent mechanism that required sustained activation of PKD1 (peaks of activation at 30 min and 240 min) to modulate the expression of markers of cardiac hypertrophy (103). In the same study, aldosterone also induced the expression of collagens and transforming growth factor-β1 in rat cardiac fibroblasts and this was dependent on the upregulation of phosphoinositide 3-kinase (PI3K)-p100delta. The MR antagonist, spironolactone, inhibited both the hypertrophic and pro-fibrotic effects, indicating an MR-dependent mechanism. Similarly, in another study it was demonstrated that aldosterone could directly stimulate cardiac myocyte hypertrophy, in a concentration-dependent manner in primary cultures of rat neonatal cardiomyocytes (104). In

this case, the aldosterone-induced hypertrophy was associated with acute activation of both ERK1/2 and PKC, within 5 minutes of aldosterone treatment. These effects were also blocked by spironolactone, indicating the involvement of MR in these responses.

Another example of non-genomic aldosterone action in cardiomyocytes is the rapid PKC epsilon–dependent induction of both $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+ pump activity in isolated rabbit cardiomyocytes (105, 106). Here, the authors found that aldosterone stimulated $Na^+/K^+/2Cl^-$ co-transport activity, leading to an increase in intracellular Na^+ concentration, with a secondary increase in pump activity within 15 min. This effect was not blocked by spironolactone or canrenone at 100-fold excess but was blocked by potassium canrenoate, the open E-ring, water-soluble MR antagonist. Activation of the cotransporter increases cardiac myocyte volume, and this may decrease cardiac compliance and hence impair ventricular filling (107). Interestingly, over-expression of PKC epsilon produced cardiac hypertrophy in transgenic mice and stimulation of PKC epsilon has been shown to induce activation of the transcription factors AP-1 and NF-kappaB (108).

The Na^+/H^+ exchanger (NHE) regulates intracellular pH via electroneutral exchange of intracellular H^+ for extracellular Na^+ and is also involved in cell volume regulation, initiation of cell growth and proliferation (109-111). Inhibition of Na^+/H^+ exchange was shown to prevent ventricular remodelling after myocardial infarction (112) and the development of myocardial hypertrophy and left ventricular dilation in a murine model of dilated cardiomyopathy (113). Aldosterone induced rapid stimulatory effects on NHE activity in VSMCs after 4 min and this effect was not blocked by canrenone (114). Here, it was shown that aldosterone stimulated an increase in intracellular inositol 1,4,5-trisphosphate (IP3) levels after 30 sec and PLC inhibition abrogated the aldosterone-stimulated Na^+ influx and increased IP3 (114). Similarly, in another study, along with genomic effects, aldosterone also induced rapid effects on NHE activity (115). The short-term effect was not mediated through MR/GR and did not depend on gene transcription or protein synthesis. Aldosterone transiently activated PKC (peak activity at 5 min), and this activation contributed to both the short- and long-term stimulatory effects of aldosterone on NHE activity (115).

MAPK is an important regulator of NHE1 isoform activity as well as cardiac hypertrophy (116, 117) and activation of NHE1 involves MR-mediated transactivation of EGFR with subsequent ERK1/2 activation (118-120). NHE1 was shown to be directly involved in aldosterone-induced hypertrophy in neonatal rat ventricular myocytes (121). In this study, aldosterone produced a significant increase in cell surface area which was inhibited by spironolactone. Although aldosterone induced MAPK1/2 activity within 5 min and decreased p38 activity within 10 min, inhibitors of these kinases had no apparent effect on the aldosterone-induced hypertrophy.

More recently, microRNAs (miRNAs) have been shown to be involved in the development of cardiac hypertrophy. miRNAs comprise a small class of non-coding RNAs that mediate post-transcriptional gene silencing and can be classified as both pro-hypertrophic and anti-hypertrophic. To silence the endogenous miRNAs that induce hypertrophic signals, engineered oligonucleotides termed “antagomirs”, that form complementary base pairs with miRNA and effectively inactivate miRNA function, have been used to antagonize cardiac hypertrophy (122-124). It was recently found that a specific microRNA, miR-9, could suppress myocardin expression in cardiac myocytes (125). Myocardin is a transcriptional cofactor which when over-expressed, can induce cardiac hypertrophy (126). Aldosterone leads to an increase in myocardin expression and miR-9 is down-regulated by aldosterone (125). The authors found that a mimic of miR-9 could attenuate cardiac hypertrophy, thus indicating the use of microRNA mimics as novel possible future therapeutics.

It is not only hypertrophy that can play a role in the progression to pathophysiological states; dysregulated apoptosis can also result in pathological consequences. The loss of cardiomyocytes through apoptosis adversely affects overall contractile function and progression to heart failure. Oxidative stress is well known to induce cardiomyocyte apoptosis either indirectly through damage to DNA, lipids or proteins or directly via the activation of pro-apoptotic signalling molecules ASK-1, JNK, ERK1/2 and p38 MAPK. In neonatal rat cardiomyocytes, aldosterone was shown to induce apoptosis by activating NADPH oxidase-mediated O_2^- production and ASK-1 (127).

4.3. Rapid aldosterone signalling in the vasculature

Aldosterone exerts multiple direct effects on the structure and function of the vasculature. In contrast to cardiac tissue, several studies have demonstrated active forms of 11-beta-HSD2 in blood vessels, in both endothelial and smooth muscle cells (128-130). Moreover, in smooth muscle cell cultures both MR and 11-beta-HSD2 are colocalized in the same cell (131). Treatment of heart failure patients with spironolactone resulted in an almost 2-fold increase in endothelium-dependent vasodilation (132-134). It was also shown that in patients with hypertension or hyperaldosteronism, 3 months of spironolactone treatment restored endothelium-dependent vasodilation, independently of any change in blood pressure (135).

Vascular smooth muscle cells (VSMC) and endothelial cells can both be regulated by aldosterone action, leading to a modulation of vasoconstriction or vasodilation, depending on the physiological context. In both VSMC and endothelial cells, aldosterone causes a rapid increase in intracellular calcium, through IP3, DAG and PKC (136-138). The consequence of these rapid effects in the vasculature depends on the bioavailability of endogenous NO (136, 138-140).

Aldosterone-induced MR-mediated vasoconstriction has been described (140, 141). Aldosterone infused into the brachial artery of healthy male volunteers, decreased blood flow significantly within 4 min

compared with the contralateral forearm, indicating rapid vasoconstrictor responses; this effect was not sustained and flow returned to baseline after ~30 min (141). Similarly, aldosterone (1-10nM) induced vasoconstriction in microperfused afferent arterioles by a mechanism involving activation of phospholipase C and calcium mobilization; this response was not inhibited by spironolactone (142).

Aldosterone-induced MR-mediated vasodilation has also been described in both rodents and humans (137, 138). Aldosterone counteracted K^+ -induced vasoconstriction within 2-5 min in microperfused rabbit renal afferent arterioles, and this effect was mediated by the classical MR and did not depend on transcription (138). Here the authors showed that inhibition of PI3-kinase restored sensitivity to K^+ in the presence of aldosterone and inhibition of NO formation by L-NAME or of soluble guanylyl cyclase restored K^+-induced vasoreactivity in the presence of aldosterone. A similar effect was also shown in rat aortic rings whereby picomolar concentrations of aldosterone counteracted the vasoconstriction induced by phenylephrine, and this effect was lost after endothelial denudation (137). In fact, in denuded vessels, aldosterone mediated a monophasic dose-dependent enhancement of the vasoconstrictor response. In this study, in cultured endothelial cells, aldosterone caused a PI3K-dependent increase in nitric oxide synthase activity as well as PI3K-dependent activation of ERK1/2 and p70 S6 kinase. At low concentrations, reactive oxygen and nitrogen species serve as physiological signalling molecules, while at higher concentrations, these are involved in pathological processes. Therefore, in conditions with low levels of oxidative stress, aldosterone may promote vasodilation, while under conditions of higher oxidative stress, aldosterone is likely to be associated with vasoconstriction and oxidative damage (18).

Aldosterone can also induce fibrotic and dysregulated proliferative effects in the vasculature and rapid signalling cascades play a central role in the initiation of these events. Aldosterone induced a rapid activation of p38 MAPK and NADPH oxidase through a c-Src–dependent pathway in VSMCs and these effects were associated with pro-fibrotic processes (143). The profibrotic action of aldosterone, assessed by determining ^{3}H-proline incorporation, a marker of collagen synthesis, was also dependent on c-Src–regulated p38 MAPK. Furthermore, non-genomic aldosterone-induced activation of c-Src, ERK1/2 and p38 MAPK was increased in spontaneously hypertensive rat (SHR) vascular cells (144). In this case, the NADPH oxidase activity, collagen synthesis, c-Src, and MAPK phosphorylation induced by aldosterone were significantly reduced by eplerenone.

Aldosterone stimulates VSMC proliferation via Big Mitogen activated protein kinase 1 (BMK1) (145). BMK1 plays a role in concentric cardiac hypertrophy and endothelial survival, in the maintenance of vascular integrity (146, 147). Here, the authors showed BMK1 was activated within 10 min in VSMCs, an effect that was independent of protein synthesis and inhibited by eplerenone. Interestingly, the anti-oxidant tiron also inhibited BMK1 activation. It was also previously shown that aldosterone-induced rapid activation of Ki-RasA, an upstream activator of the BMK1 cascade, was attenuated by spironolactone (148).

The signalling mechanisms of aldosterone and angiotensin are often closely intertwined and there is increasing evidence that aldosterone acting via MR may mediate or exacerbate the damaging effects of angiotension II. For example, the AT_1R antagonist losartan prevented collagen accumulation in the aldosterone-salt model, indicating cross-talk between AT_1R and MR activation (80). Furthermore, in rat arterial smooth muscle cells (RASMC) and human vascular smooth muscle cells, aldosterone enhanced angiotensin II-induced protein synthesis (14, 149) and both aldosterone and angiotensin II induced cardiac fibrosis, characterized by enhanced accumulation of collagen and increased fibroblast proliferation *in vivo* (82, 150, 151). It was also previously demonstrated that aldosterone treatment increased angiotensin II receptor expression and enhanced the intracellular signalling to promote cell growth in vascular smooth muscle cells (152) and angiotensin II-stimulated RASMC proliferation was blocked by spironolactone (153).

The proliferation of VSMCs was stimulated by a combination of very low concentrations of aldosterone (10^{-12} mol/L) and angiotensin II (10^{-10} mol/L), while aldosterone or angiotensin II alone had no effect at these concentrations (154). This effect was inhibited by blocking the AT_1 receptor, MAPK1/2 signalling, EGFR or MR activation using spironolactone. Aldosterone and angiotensin II produce a synergistic bimodal ERK activation at 10-15 min and at 2-4 hrs. The early phase of ERK activation was markedly inhibited by an AT_1 receptor inhibitor but not by spironolactone, whereas the later phase of ERK activation was attenuated by both inhibitors, as well as by actinomycin D and cyclohexamide. The rapid ERK activation associated with EGFR transactivation in response to aldosterone and angiotensin II has an impact on the genomic induction of Ki-ras2A, and the down-regulation of the mitogen-activated protein kinase phosphatase-1, MKP-1 (154). Aldosterone and high salt intake (Ald-NaCl) increased cardiac fibrosis in angiotensin receptor 1a knockout mice (AT1aR-KO), but not in wild-type mice, showing that aldosterone-salt can induce cardiac fibrosis independently of the angiotensin receptor signalling pathway (155). Interestingly, the MR antagonist eplerenone prevented cardiac fibrosis, Rho kinase phosphorylation and oxidative stress in aldosterone-salt treated AT1aR-KO mice (156). Co-operation between aldosterone and angiotensin II has been identified in the release of free radicals that can lead to deterioration of arterial smooth muscle cells (157). Aldosterone alone induced the activation of JNK and ERK1/2 after 10 min and potentiated angiotensin II-induced signalling in VSMCs (rapid ERK1/2 phosphorylation and JNK phosphorylation after 2 min). This stimulation was dependent on ROS generation and inhibiting EGFR attenuated the ERK1/2 signalling induced by aldosterone or angiotensin (157).

5. ALDOSTERONE AND THE BRAIN

Aldosterone has a multitude of effects on brain function. MR is expressed in the neurons of the CNS, with the highest abundance found in the limbic system, in particular within the hippocampus (158). Some parts of the brain that regulate salt appetite also express 11-beta-HSD2 and therefore are aldosterone-sensitive. For example, the colocalization of MR and 11-beta-HSD2 was demonstrated in discrete neurons of the *nucleus tractus solitarii* using immunohistochemistry (159) and these neurons showed an increase in nuclear-localized MR after aldosterone infusion in adrenalectomized rats (160). The functions of aldosterone in the regulation of mammalian salt, water and acid-base balance are mediated by the aldosterone-induced stimulation of regions within the amygdala and hypothalamus involved in salt appetite and osmotic regulation, respectively (161, 162). Aldosterone actions within the central nervous system have been shown to result in both acute and long-term effects on vascular structure and function and to result in hypertension (163). Activation of MR by aldosterone in the amygdala increases salt appetite and sodium intake, greatly exacerbating hypertension and associated end-organ disease (164).

However, the remaining parts of the CNS do not express 11-beta-HSD2, leaving MR "unprotected" and open to activation by the abundant circulating glucocorticoids. MR has been shown to have a 10-fold higher affinity for corticosterone than GR in the brain (165). It is therefore likely that MR will be occupied by corticosterone in most parts of the CNS. An additional mechanism other than 11-beta-HSD2 to allow aldosterone to compete successfully with corticosterone for MR in target cells of the brain could be the local synthesis of aldosterone in or near aldosterone target neurons. Supporting this theory, the required enzymes for aldosterone synthesis from cholesterol are expressed in the normal human and rat brain (13, 166-169) and low levels of aldosterone are synthesized in normal rat brain *in vitro* and *in vivo* (13, 166, 170). However, the amounts of extra-renal aldosterone are extremely low compared to adrenal production and any physiological role for this minor local synthesis of aldosterone remains to be clarified.

Both MR and GR are co-expressed in many cells of the brain, including the Purkinje cells of the cerebellum and hippocampus (167, 171). If both receptors are expressed together, they may mediate the corticosteroid signal in synergism or antagonism, depending on the cellular context (172). Moreover, as another layer of complexity, heterodimerization of the two receptors appears to be important in the transcriptional regulation of the glucocorticoid-responsive genes in the brain (173, 174).

5.1. MR and GR in learning and memory

The hippocampus is critical for learning and memory processes, particularly regarding spatial orientation. High levels of both MR and GR are expressed in the dentate gyrus of the hippocampus (175). Previous studies have shown that GR and MR play different roles in cognitive behaviour; blockade of either GR or MR alters diverse aspects of spatial learning and memory (176). The glucocorticoid receptor is involved in the consolidation of recently acquired information, whereas the mineralocorticoid receptor is essential for the interpretation of environmental stimuli and selection of appropriate behavioural responses. Adrenalectomized rats showed deficiency in both MR- and GR-mediated function, showing impaired place navigation learning as well as altered search strategies (176).

Corticosteroids induce non-genomic effects in the brain, acting via MR. Corticosterone caused a rapid and reversible enhancement of the frequency of mEPSCs (miniature excitatory post-synaptic currents) in CA1 pyramidal cells in adult mouse hippocampal slices (177). This increase in frequency was not inhibited by the protein synthesis inhibitor cycloheximide nor by the GR antagonist RU 486. In the same study, aldosterone, markedly enhanced mEPSC frequency (177), an effect which was completely blocked by spironolactone. Furthermore, no effect of corticosterone on mEPSC frequency was observed in CA1 cells of forebrain-specific MR knockout mice as opposed to the controls (177). The increased mEPSC frequency was found to involve a presynaptically located MR, which caused fusion of glutamate-containing vesicles with the presynaptic membrane through the activation of the ERK1/2 pathway (178). In the presence of U0126 (a selective MEK inhibitor- an upstream activator of ERK1/2), corticosterone failed to enhance mEPSC frequency (178).

5.2. The opposing effects of GR and MR on neuronal survival

In rats, chronic systemic infusions of aldosterone increased anxiety-like behaviour (179), and in humans large amounts of aldosterone caused a state of "malaise" that lacked any apparent physiological basis (180). In humans, elevated aldosterone production, particularly at night, has been identified in association with depression (181, 182). Primary aldosteronism, a disease characterized by the overproduction of aldosterone has also been linked to an elevated rate of generalized anxiety disorder (183). Depressive symptoms, the presenting complaint of some patients with hyperaldosteronism, may remit after spironolactone treatment or unilateral adrenalectomy (184, 185).

It has been proposed that activation of MR stimulates neurogenesis in the dentate gyrus of the adrenalectomized animal (186). However, contradictory findings suggesting that MR activation suppresses proliferation have also been reported (187). Hippocampal cell loss is likely to affect cognition and the regulation of mood and anxiety. The loss of neurons expressing MR and GR in the hippocampus is a primary cause of disinhibited hypothalamic-pituitary-adrenal axis (HPA) activity. MR is thought to be tonically activated under basal levels of HPA activity, whereas GR becomes occupied by corticosteroids in the high physiological range. GR activation stimulates a molecular cascade, leading to significant levels of neuronal cell death through apoptotic mechanisms (188), and MR activation counteracts the deleterious effects of

glucocorticoids on neuronal survival (188-190). Moreover, MR occupation may be essential for dentate granule neuron survival (191-193). In primary hippocampal cell cultures, a protective effect of MR activation has been shown in GR-induced apoptosis (194). In this study, the GR agonist dexamethasone doubled the amount of apoptotic cells within 48h, whereas aldosterone significantly attenuated the apoptosis-inducing actions of dexamethasone. This was consistent with previous findings (188, 189) from the same group that MR occupation counteracts the actions of GR. Moreover, blockade of MR with spironolactone resulted in a dose-dependent increase in the rate of apoptosis, indicating that tonic occupation of MR is essential for neuronal survival.

The opposing effects of GR and MR on neuronal survival result from their ability to differentially influence the expression of members of the *bcl-2* gene family (188). Specifically, in the rat hippocampus, activation of GR induces cell death by increasing the ratio of the pro-apoptotic molecule Bax relative to the anti-apoptotic molecules Bcl-2 or Bcl-x_L, and the opposite effect was observed after MR activation. Moreover, GR activation increases and MR activation decreases the levels of tumor suppressor protein p53 (a direct transcriptional regulator of *bax* and *bcl-2* genes). Dexamethasone induced a rapid translocation of the tumor suppressor p53 to the nucleus within 30 min of treatment in a neural cell line HT22, enhanced the transcription of p53-responsive genes and increased the transactivation potential of exogenous p53 (195).

6. CONCLUSION

In this review, we have outlined the impact of rapid aldosterone-mediated responses on the deleterious processes of dysregulated proliferation, hypertrophy and apoptosis, how these eventually lead to pathophysiological states such as chronic kidney disease and heart failure, and how selective antagonists can ameliorate these effects by impacting on both the initial rapid signalling events and the later transcriptional responses. Overall it is clear that aldosterone not only plays a central role in controlling salt and water homeostasis, and therefore blood pressure, but also mediates direct effects on tissue structure and function in epithelial tissues such as the kidney and at non-epithelial sites such as the heart, vasculature and brain. This dual mode of action likely results in the exacerbation of disease progression in certain pathologies. Therefore, blocking the effects of aldosterone using specific antagonists of the endogenous receptor, MR, has beneficial effects not only on the regulation of blood pressure but also on the regulation of cell proliferation, hypertrophy and apoptosis in various disease states.The rapid and genomic effects of aldosterone are multi-faceted and extremely complex, depending on the local concentration of the hormone and the nature of the local cellular environment. The challenge remains for future aldosterone research, to understand how the rapid non-genomic extra-nuclear signalling events are integrated with the later genomic responses, to ultimately lead to the final physiological or pathophysiological outcome.

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