Mineralocorticoid regulation of cell function: the role of rapid signalling and gene transcription pathways

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Abstract

The mineralocorticoid receptor (MR) and mineralocorticoids regulate epithelial handling of electrolytes, and induces diverse effects on other tissues. Traditionally, the effects of MR were ascribed to ligand–receptor binding and activation of gene transcription. However, the MR also utilises a number of intracellular signalling cascades, often by transactivating unrelated receptors, to change cell function more rapidly. Although aldosterone is the physiological mineralocorticoid, it is not the sole ligand for MR. Tissue-selective and mineralocorticoid-specific effects are conferred through the enzyme 11β-hydroxysteroid dehydrogenase 2, cellular redox status and properties of the MR itself. Furthermore, not all aldosterone effects are mediated via MR, with implication of the involvement of other membrane-bound receptors such as GPER. This review will describe the ligands, receptors and intracellular mechanisms available for mineralocorticoid hormone and receptor signalling and illustrate their complex interactions in physiology and disease.

Key Words
- aldosterone
- mineralocorticoid receptor
- glucocorticoid
- MAPK pathways
- GPER
- EGFR

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Introduction

The mineralocorticoid receptor (NR3C2, henceforth abbreviated MR) and mineralocorticoids regulate numerous physiological processes including control of electrolytes, extracellular volume and blood pressure (Waldinger *et al*. 1977), intracellular pH (Oberleithner *et al*. 1987), cardiac action potentials (Lalevee *et al*. 2005, Boixel *et al*. 2006) and vascular function (Liu *et al*. 2003, Uhrenholt *et al*. 2003, Gros *et al*. 2013) among others. The MR also contributes to cardiovascular and renal disease. Primary hyperaldosteronism (PA), a cause of secondary hypertension due to chronic excessive aldosterone synthesis, is associated with increased mortality and morbidity independent of the degree of hypertension (Milliez *et al*. 2005).

The MR can change cell function through multiple means. Edelman first proposed that aldosterone modifies sodium transport via gene transcription (Edelman *et al*. 1963), a mechanism recently confirmed as critical for life-sustaining salt homeostasis (Cole *et al*. 2015). MR activation also triggers rapid responses that are impervious to transcription inhibitors, suggesting a non-genomic action (Moura & Worcel 1984, Le Moellic *et al*. 2004). Furthermore, mineralocorticoids may activate receptors other than the 'classic' cytosolic MR: either as a ligand of a different cell membrane-associated receptor, or by influencing signalling of unrelated receptors such as angiotensin II receptor 1 (AGTR1). These systems do not occur in isolation, and may enable, complement,

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augment or abrogate each other. Given the complexity at several levels, the purpose of this review is to identify the key mechanisms of mineralocorticoid action and characterise their actions and contribution to physiology and disease.

The structure and function of the MR

In humans, the MR is part of a steroid-activated transcription factor superfamily and retains significant structural similarities to the glucocorticoid receptor (NR3C1, henceforth abbreviated GR) and progesterone receptor (PGR) (Arriza *et al*. 1987). All nuclear receptors contain an amino terminal domain (NTD), DNA-binding domain (DBD), hinge region and a ligand-binding domain (LBD). The MR and other steroid hormone receptors are activated through ligand–LBD interaction, but other parts of their structure can affect outcomes. The NTD, via its activation function-1 (AF-1a and AF-1b) sites, interact with nuclear proteins, and, together with AF-2 sites in the LBD, can bind co-regulatory molecules which serve to modify transcriptional function (Pippal & Fuller 2008).

In the basal or unliganded state, the MR is located predominantly in the cytosol (Rogerson *et al*. 2004) as part of a heterocomplex with chaperone heat shock proteins (HSPs) such as HSP90, immunophilins (such as FKBP52) and protein phosphatase 5 (Galigniana *et al*. 2010*a*, Huyet *et al*. 2012). HSP90 facilitates ligand binding to MR, while FKBP52 is important in cytoplasmic–nuclear shuttling of MR after ligand binding (Galigniana *et al*. 2010*b*). Once in the nucleoplasm, the MR dissociates from its chaperones to allow binding to DNA (Galigniana *et al*. 2010*a*) and forms dimers (Nishi *et al*. 2004, Grossmann *et al*. 2012). The MR not only forms homodimers, but also heterodimerises with GR, resulting in different transcriptional responses. The degree of heterodimerisation depends on the relative abundance of activated MR and GR, which is influenced by hormone availability, cell-specific steroid handling and receptor expression (Nishi *et al*. 2004, Ackermann *et al*. 2010, Nishi 2011).

The MR DBD binds to specific DNA sequences, known as hormone response elements (HREs), to regulate transcription of target genes (Fig. 1, section A). The HREs could also bind GR; they were originally described in that context (Payvar *et al*. 1983). The crystal structure of the MR DBD when bound to a HRE is similar to that for the GR (Hudson *et al*. 2014). The HRE structure allows each receptor in the dimer to bind to a 'half-site' of the palindromic consensus sequence (Nishi *et al*. 2004, Grossmann *et al*. 2012). In many HREs, sequences adjacent to the consensus motifs facilitate binding of non-hormone transcription factors such as activated protein-1 (AP-1), early growth response protein 1 (EGR1), forkhead box (FOX) and paired box protein 5 (PAX5) (Pearce & Yamamoto 1993, Le Billan *et al*. 2015). Interaction with co-factors at the NTD may explain some of the differences in gene regulation between MR and GR despite the overlap in ligand binding, receptor structure and target DNA sequence recognition (Pearce & Yamamoto 1993, Lim-Tio *et al*. 1997). Some HREs can preferentially enhance transcription in response to MR than GR (Kolla *et al*. 1999), or may only bind MR specifically (Meinel *et al*. 2013*b*). As the MR can bind to many areas of DNA which lack a partial or full classical HRE sequence, HREs are not mandatory for MR genomic regulation. Instead, MR may form complexes with other transcription factors that have known binding sites in these HRE-free regions, rather than directly binding DNA itself (Le Billan *et al*. 2015).

Pre-receptor and receptor mechanisms determining ligand-specific effects of MR

Aldosterone is the major physiological mineralocorticoid and its importance is demonstrated by the neonatal onset of life-threatening salt wasting and hyperkalaemia when it is deficient (Daughaday & Rendleman 1967, Hui *et al*. 2014). Aldosterone is synthesised in the zona glomerulosa of the adrenal gland under the regulation of the renin–angiotensin system (RAS), extracellular potassium levels and adrenocorticotropic hormone (ACTH). Its function in regulating salt and fluid balance is achieved by altering the sodium transport machinery of renal tubular epithelial cells (Loffing & Korbmacher 2009) and is critical for protection against hypovolaemia (Fine *et al*. 1958, Beuschlein 2013).

Apart from aldosterone, the human MR has high affinity for the glucocorticoids cortisol and corticosterone (Pearce & Funder 1988), and the sex steroid progesterone (Quinkler *et al*. 2002). This may be a vestige of evolution, with progression from a single multifunctional corticosteroid receptor (CR) in primitive marine animals to distinct mineralocorticoid and glucocorticoid hormones and receptors in higher-order land animals (Baker *et al*. 2013). Given that glucocorticoids are substantially more abundant than mineralocorticoids in the circulation and intracellular fluid, mechanisms must exist to confer specificity of

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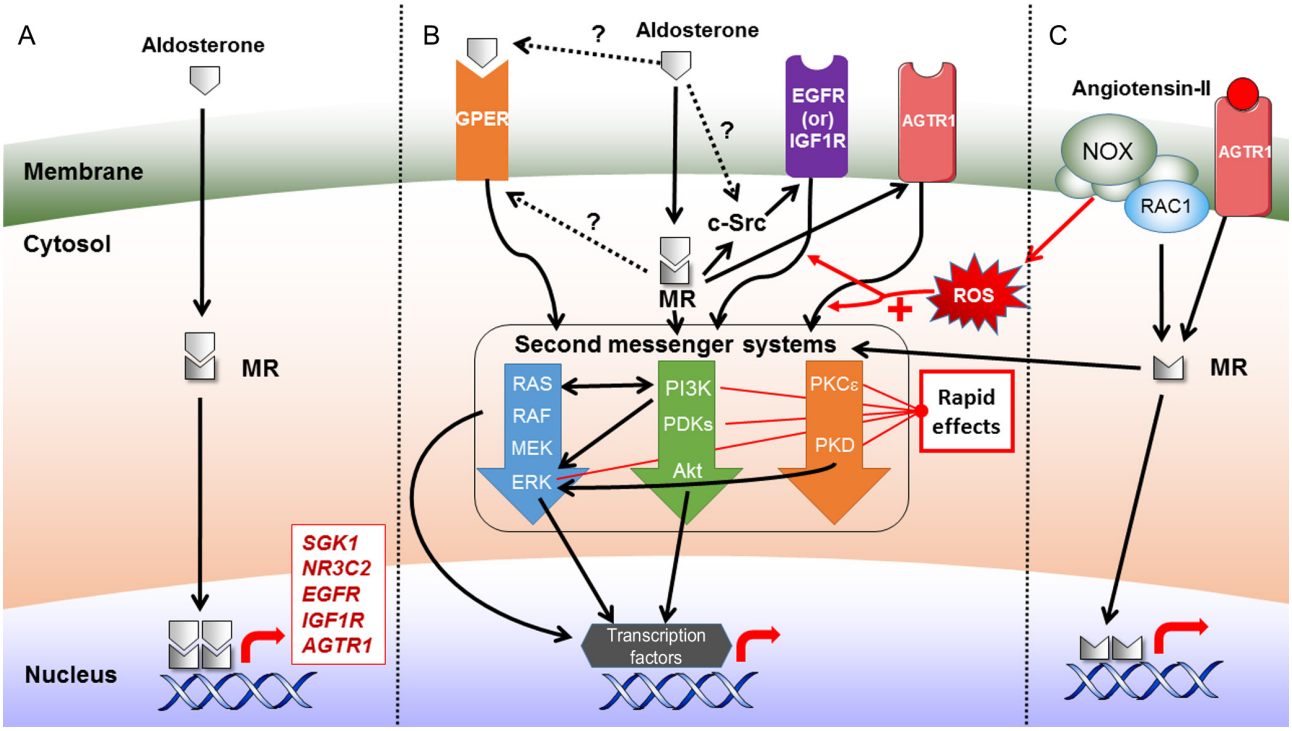

Figure 1
Overview of the cellular responses to MR activation. (A) Once activated, the mineralocorticoid receptor (MR) translocates to the nucleus and transcribes target genes including *SGK1*, sodium channel subunits and receptors such as the MR gene (*NR3C2*), epidermal growth factor receptor (*EGFR*), insulin-like growth factor 1 receptor (*IGF1R*) and angiotensin II receptor 1 (*AGTR1*). Some gene targets are intermediaries that activate other transcription factors. (B) Alternatively, aldosterone and MR act via second messenger systems, often by activating unrelated receptors in the absence of their ligands. Note, the MR may also signal via other MAPK cascades (JNK and p38MAPK) but only the ERK cascade is shown here. Additionally, aldosterone may act in an MR independent manner – potentially by directly binding to and activating unknown membrane receptors instead. GPER is one such proposed receptor, although MR binding to it has not been definitively demonstrated. (C) RAC1 and AGTR1 can also activate MR signalling and gene transcription in certain circumstances, in the absence of any ligand binding to MR. Some signalling pathways are redox sensitive and enabled or enhanced by NADPH oxidase (NOX) production of reactive oxygen species (ROS).

effect at the MR. The enzyme 11-beta-hydroxysteroid dehydrogenase type 2 (HSD11B2) is co-expressed with MR in epithelial cells, metabolising cortisol to cortisone, which cannot bind or activate the MR (Funder *et al.* 1988). This is crucial for the specificity of aldosterone as the regulator of fluid homeostasis. If HSD11B2 is deficient or inhibited, hypertension and hypokalaemia develop due to cortisol activation of renal MR (Koster & David 1968, Dave-Sharma *et al.* 1998, Mullins *et al.* 2015). In the vasculature, endothelial cells can express HSD11B2 (Brem *et al.* 1998, Christy *et al.* 2003, Gong *et al.* 2008), while the literature is conflicting regarding its presence in vascular smooth muscle cells (VSMCs) (Hatakeyama *et al.* 2001, Christy *et al.* 2003). Deficiency or inhibition of HSD11B2 impairs endothelium-mediated vasodilation, but glucocorticoid occupation of MR may not be the cause (Christy *et al.* 2003, Sobieszczyk *et al.* 2010). Instead, a potential mechanism may involve regulation of endothelial nitric oxide synthase (eNOS) expression. Glucocorticoids inhibit eNOS transcription, which is exacerbated by HSD11B2 knockdown in a human umbilical vein endothelial cell (HUVEC) line (Liu *et al.* 2009).

Generally, in tissues where HSD11B2 is not expressed, glucocorticoids are the physiological ligand for the MR (Iqbal *et al.* 2014). An exception occurs where the related enzyme HSD11B1 is expressed, without co-expression of hexose-6-phosphate dehydrogenase (H6PD). H6PD generates the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), without which HSD11B1 shifts its role from reductase to dehydrogenase, metabolising cortisol to cortisone (Hewitt *et al.* 2005, Chen *et al.* 2014). Transgenic mice overexpressing HSD11B2 in cardiomyocytes develop dilated cardiomyopathy and fibrosis, which is attenuated with MR antagonism (Qin *et al.* 2003), implying that basal cardiac MR occupancy by endogenous glucocorticoid is protective against activation. However, in situations of

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intracellular oxidative stress, such as with inflammation or ischaemia, glucocorticoids can activate the MR (Rossier *et al*. 2008, Mihailidou *et al*. 2009). Therefore, the benefits of spironolactone or eplerenone in heart failure are not solely from aldosterone antagonism, but general blockade of MR (Pitt *et al*. 1999, 2003, Zannad *et al*. 2011). Hence, the availability of ligand, distribution of HSD11B2 expression and the redox status of cells determine the final response of the MR to ligand activation.

The intrinsic properties of the MR structure influence the outcome to ligand binding at the receptor. The MR LBD pocket shape and van der Waal forces between residues on the LBD and steroid determine ligand binding affinity and transcriptional activity (Li *et al*. 2005, Mani *et al*. 2016). After ligand binding, the MR changes conformation and recruits other elements to facilitate nuclear localisation and transcription (Yang & Young 2009). Aldosterone remains bound to MR for a comparatively longer period than cortisol, which stabilises the MR in a conformation that can more effectively recruit co-regulators, resulting in aldosterone having greater potency than cortisol for inducing MR target gene transcription (Hellal-Levy *et al*. 2000, Gallo *et al*. 2007, Huyet *et al*. 2012). It is also possible for MR activation and nuclear translocation to occur without ligand binding. Ras-related C3 botulinum toxin substrate 1 (RAC1) is part of the Rho family of small GTPases, which regulate many cellular processes. Importantly, it is involved in ROS generation and can activate steroid hormone receptors including the MR (Shibata *et al*. 2008). RAC1 activation, with associated ligand-independent MR activation, can occur in the context of oxidative stress (Nagase *et al*. 2012) (Fig. 1, section C), salt loading in salt-sensitive Dahl rats (Shibata *et al*. 2011), and in a transverse aortic constriction model of cardiac pressure overload (Ayuzawa *et al*. 2015). Further work is required to confirm these RAC1 mechanisms, and establish the molecular link with MR overactivity.

How the activated MR changes cellular processes

Expression of MR target genes

The MR utilises several mechanisms to effect cellular change. These mechanisms allow diversity in timing, duration, magnitude and context or nature of the effect (Fig. 1). MR regulates the transcription of many genes (Fig. 1, section A), with well-established MR targets being those related to electrolyte handling in renal epithelial tissues, such as sodium channels or transporters (Mick *et al*. 2001). Further candidate MR target genes are proposed through transcriptome analyses on renal, aortic and cardiac tissue after exposure to aldosterone. These have diverse functions in cell signal transduction, oxidative stress, inflammatory mediators, steroid biosynthesis, receptor chaperoning, cellular structure, adhesion and migration (Turchin *et al*. 2006, Latouche *et al*. 2010, Newfell *et al*. 2011, Ueda *et al*. 2014). Comparison between mice with cardiac overexpression of either GR or MR suggests that there is surprisingly little overlap in GR- and MR-regulated genes in the heart (Latouche *et al*. 2010). Novel genes identified in these experiments require further investigation to establish the mechanism and functional outcomes of their regulation by MR.

Rapid signalling through second messenger systems

Gene transcription and protein translation is a relatively slow process. A delay of several hours may transpire before any functional change, if protein synthesis, export, translocation and assembly is required (such as for membrane-based sodium channels). This would be inadequate when a rapid homeostatic response to acute disturbance is required, such as during haemorrhage. MR activation can trigger more rapid cellular events through non-genomic means. For instance, aldosterone increases epithelial sodium channel (ENaC) activity within 2 min (Zhou & Bubien 2001), which is significantly faster than the 30 min required for mRNA expression of serum and glucocorticoid-regulated kinase 1 (*SGK1*), a 'rapidly' transcribed MR target gene (Naray-Fejes-Toth & Fejes-Toth 2000). The MR is able to utilise second messenger systems to initiate these rapid effects (Fig. 1, section B).

Mitogen-associated protein kinases (MAPK) MAPKs are a group of serine/threonine cytoplasmic protein kinases, which catalyse phosphorylation and activation of proteins to regulate numerous diverse cellular processes. As a cascade of sequentially activated kinases, MAPK relays signals from the cell surface (e.g. from a membrane receptor) to the interior (Roskoski 2012). In mammals, the key families of MAPK are extracellular signal-regulated kinase (ERK), p38 kinase (p38 MAPK) and c-jun N-terminal kinase (JNK) all of which can be triggered by MR activation (Nagai *et al*. 2005, Han *et al*. 2009, Walczak *et al*. 2011). MAPK signalling is important for MR-mediated cell proliferation or apoptosis, such as in the developing neonatal rat kidney (Yim *et al*. 2009), and cellular electrolyte handling (Gekle *et al*. 2001,

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McEneaney *et al*. 2008). The ERK cascade (RAS-RAF-MEK-ERK) is rapidly activated within 2–5 min by aldosterone (Gekle *et al*. 2001, McEneaney *et al*. 2010*a*); JNK can similarly be activated within 5 min (Han *et al*. 2009), and p38 MAPK within 10 min (Lee *et al*. 2004). Initial rapid ERK1/2 activity lasts around 30 min (Nagai *et al*. 2005), but can be extended to around 2 h in a protein kinase D (PKD)-dependent mechanism (McEneaney *et al*. 2010*a*), with further prolongation of the response to 4–6 h requiring transcription of Kirsten Ras (K-Ras) mRNA (Hendron & Stockand 2002). Less is known about prolonged activation of the other MAPK cascades by MR.

Phosphatidylinositide lipid and protein kinase messenger system Phosphatidylinositide 3-kinases (PI3K) activity is stimulated by aldosterone, which phosphorylates membrane phosphatidylinositol and generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Blazer-Yost *et al*. 1999). PIP3 is required to activate phosphatidylinositol-dependent kinases (PDK) and ultimately Akt as the effector of PI3K-dependent cellular processes (Ghigo & Li 2015). MR-dependent Akt phosphorylation occurs within 15 min of aldosterone exposure, suggesting that PI3K/Akt is a pathway for MR-mediated rapid effects (Huang *et al*. 2012) including electrolyte handling and vasomotor function.

Protein kinases C (PKC) and D PKC and PKD form part of a regulatory signalling cascade, commonly under the regulation of G-protein coupled surface receptors. Activation of the PKCε subtype leads to phosphorylation of PKD at two critical activating sites leading to downstream effects including membrane trafficking, cell survival, cell migration and interaction with MAPK cascades (Rozengurt *et al*. 2005). MR uses PKC and PKD signalling to alter electrolyte handling in renal epithelial cells and in cardiomyocytes (Mihailidou *et al*. 2004, McEneaney *et al*. 2008).

Interaction with other hormone receptor systems

The intracellular signalling cascades induced by MR activation are complex and intricately intertwined. Mapping discrete pathways linking MR to cellular outcomes is difficult due to the extent of cross-talk between the elements, and their occasionally opposing effects. This difficulty is further exacerbated when considering the involvement of other receptor systems in this process. In many cases, second messenger systems are not directly activated by MR. Instead the MR *transactivates* other receptors, which trigger downstream signalling similar to activation by their own ligand. Although these transactivated receptors share second messenger systems, their effects are not identical due to differences in receptor expression and the specific context required for activation (particularly redox status). These effects span the full time course of cellular events from rapid posttranslational modifications to slower gene transcription (Wang *et al*. 2001, Holzman *et al*. 2007, Cascella *et al*. 2010).

Epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) The EGFR is a transmembrane receptor tyrosine kinase, which, along with structurally similar receptor tyrosine kinases such as HER2, ErbB3 and ErbB4, is part of the ErbB family. When activated, EGFR homodimerises or heterodimerises with another member of the ErbB family, triggering autophosphorylation of tyrosine residues in its cytoplasmic domains and activation of associated intracellular signalling cascades (Mirone *et al*. 2015). These include MAPK, Janus kinase/signal transducers and activators of transcription (JAK/STAT) and PI3K/Akt (Mirone *et al*. 2015). EGFR, a mediator of growth and repair, is a recognised contributor to renin–angiotensin–aldosterone system (RAAS)-driven cardiac and renal fibrosis (Zhuang & Liu 2014, Forrester *et al*. 2016). Aldosterone activates EGFR in a non-MR-dependent process within 10 min, triggering the ERK cascade and ultimately causing calcium influx and cellular alkalinisation through increased activity of the sodium/hydrogen exchanger (NHE)-1 (Gekle *et al*. 2002). Aldosterone activation of other multifunctional signalling pathways via EGFR, such as the JNK pathway (Grossmann *et al*. 2005) and PI3K/Akt (Huang *et al*. 2009), are MR dependent. The signalling process is influenced by the cellular redox state, in that the antioxidant N-acetylcysteine (NAC) prevents downstream effects of aldosterone–MR transactivation of EGFR on PI3K (Huang *et al*. 2009).

As illustrated in Fig. 1 section B, the link between MR and EGFR activation is the non-receptor tyrosine kinase, c-Src, which phosphorylates a tyrosine residue at position 845 on EGFR (Grossmann *et al*. 2005, McEneaney *et al*. 2007). Aldosterone rapidly increases c-Src phosphorylation within 5 min and has maximal response at 30 min (Callera *et al*. 2005). Furthermore, c-Src activation by MR may be dependent on the PDGFR in a complex interaction occurring within cellular invaginations, termed caveolae. Here, the transactivation of PDGFR by MR facilitates translocation of c-Src to cholesterol-rich domains and its phosphorylation (Callera *et al*. 2011*b*). Another potential

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link is the G-protein coupled oestrogen receptor (GPER), which is required for MR–EGFR transactivation at least in one ER-negative breast cancer cell line (Rigiracciolo *et al.* 2016). Furthermore, there is a synergistic relationship between MR and the EGFR. As an MR target gene, EGFR expression is upregulated by MR activation (Krug *et al.* 2003, Meinel *et al.* 2013*a*). Conversely, EGFR activation of ERK1/2 signalling is an important facilitator of MR nuclear shuttling (Grossmann *et al.* 2005). These complementary events could potentiate EGFR-related signalling from prolonged MR activation.

Insulin-like growth factor-1 receptor (IGF1R) The IGF1R is ubiquitously expressed and is important in the regulation of cell growth mainly through MAPK signalling, and metabolism through PI3K/Akt signalling. Its primary ligand, IGF-1, is not only important as the effector protein of the growth hormone system, but is involved in cardiovascular function, insulin resistance and pancreatic beta islet cell function and malignancy (Abbas *et al.* 2008). Aldosterone induces phosphorylation of IGF1R within 10 min in renal and cardiac fibroblasts, and in renal epithelial cells (Bunda *et al.* 2007, Holzman *et al.* 2007, Chen *et al.* 2013). In fibroblasts, aldosterone does not require MR to transactivate IGF1R, but utilises c-Src as an intermediary (Chen *et al.* 2013). The activation of c-Src in fibroblasts may depend on a surface membrane G-protein coupled receptor, as siRNA knockdown of the G-protein subunit $G\alpha_{13}$ prevented c-Src and IGF1R phosphorylation (Bunda *et al.* 2009). In renal epithelia, IGF1R transactivation requires MR, but the mechanism is not yet characterised (Holzman *et al.* 2007). As IGF-1 can mimic some aldosterone effects on renal sodium handling via PI3K, and can activate similar second messenger systems to MR, the IGF1R is a candidate intermediary for MR action (Blazer-Yost *et al.* 1999). IGF1R expression can be upregulated by MR, particularly in conditions of oxidative stress, with enhanced downstream signalling promoting VSMC growth, migration and protein synthesis (Cascella *et al.* 2010).

Angiotensin II receptor 1 (AGTR1) Angiotensin II is an important effector protein of the RAAS system and a major secretagogue for aldosterone. It acts primarily through two receptors: AGTR1 and AGTR2. AGTR1 is associated with classical functions ascribed to angiotensin II such as vasoconstriction, reactive oxygen species (ROS) generation, vascular cell proliferation, aldosterone production, salt/fluid retention and increased sympathetic activity. AGTR2 has opposing effects including vasodilation, nitric oxide (NO) generation and promotion of apoptosis (Vinturache & Smith 2014). Both AGTR1 and MR play a role in rapid signalling triggered by mineralocorticoids and angiotensin II. In mouse mesenteric vessels, aldosterone-induced ERK activation and rapid vasoconstriction requires AGTR1, but is MR independent (Yamada *et al.* 2008, Lemarie *et al.* 2009). However, AGTR1 and MR are both required for activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription complex which regulates numerous inflammatory genes (Lemarie *et al.* 2009). As angiotensin II also requires MR for NF-κB activation, the cross-talk between MR and AGTR1 is a common molecular signalling mechanism spanning both ligands (Lemarie *et al.* 2009). However, the nature of the MR-AGTR1 interaction varies between cell types: for example, in contrast to vascular cells, aldosterone-induced ERK phosphorylation needs both MR and AGTR1 in cardiomyocytes (Cannavo *et al.* 2016).

In rodents, AGTR1 occurs as two subtypes (a and b) which have differing effects on downstream signalling pathways. In mouse mesenteric VSMCs, angiotensin II and aldosterone activation of ERK1/2 and JNK was AGTR1a but not AGTR1b or MR dependent, but both AGTR1 subtypes are needed for NF-κB activation (Lemarie *et al.* 2009). AGTR1a has also been identified as an important facilitator of aldosterone-mediated genomic effects. Knockout of AGTR1a reduces transcription of c-fos, a rapidly induced transcription factor, in response to aldosterone compared to wild type in VSMCs (Lemarie *et al.* 2009). The relevance of AGTR1 subtypes to humans is unclear, with little in the literature regarding their existence and whether they are analogous to the mouse subtypes (Konishi *et al.* 1994).

As with EGFR/PDGFR and IGF1R, c-Src is an important link between MR, AGTR1 and the ERK cascade (Cannavo *et al.* 2016). In fact, EGFR/PDGFR signalling with activation of c-Src can be triggered by synergism of angiotensin II and aldosterone at low doses that individually do not alter cell signalling. Downstream processes are also activated, such as generation of ROS by NADPH oxidase (NOX), translocation of RhoA/Rho kinase to the cell membrane, ROS-dependent activation of RhoA and finally VSMC migration (Montezano *et al.* 2008). Hence, in the correct environment and cell context, c-Src links MR and multiple other receptor signalling pathways. Apart from the contribution of c-Src, exactly how mineralocorticoids and the MR could transactivate AGTR1 is unknown. Aldosterone triggers dimerization of AGTR1, with the transglutaminase enzyme as a critical intermediary (Yamada *et al.* 2008); given

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that transglutaminase activity is calcium-dependent, aldosterone-induced calcium influx may be an early regulator of AGTR1 transactivation. It is not clear if this is an MR-dependent effect or not. Further research is needed to confirm this theory and to characterise the remaining components of the pathway.

The relationship among aldosterone, angiotensin II, MR and AGTR1 serves to mutually enhance the signalling of each individual ligand–receptor system. Aldosterone is able to upregulate the expression of both MR and AGTR1 (Schiffrin *et al*. 1985, Zennaro *et al*. 1996, Tsai *et al*. 2013). In cardiomyocytes, aldosterone control of MR expression is dependent on MR coupled to AGTR1 signalling and downstream ERK and JNK activation, whereas AGTR1 expression is regulated by MR-independent transactivation of AGTR1 signalling (Tsai *et al*. 2013). Furthermore, aldosterone activation of MR increases transcription of angiotensin-converting enzyme (*ACE*) mRNA in the aorta of rats treated with aldosterone (Hirono *et al*. 2007) and in cultured rat aortic endothelial cells (Sugiyama *et al*. 2005). This process is JAK2 dependent and requires downstream c-Src signalling and transactivation of EGFR. The resultant increase in local angiotensin II levels exacerbates endothelial dysfunction and damage (Sugiyama *et al*. 2005). ACE expression in cardiomyocytes is similarly enhanced by MR (Harada *et al*. 2001, Wang *et al*. 2002). However, the practical relevance of local ACE activity to vasomotor function is uncertain given that aldosterone-induced mesenteric vasoconstriction *ex vivo* is not mitigated by ACE inhibition (Yamada *et al*. 2008).

In a bilateral relationship, angiotensin II can transactivate MR via AGTR1 and increase transcription of MR-dependent genes, a process that can be suppressed by spironolactone (Jaffe & Mendelsohn 2005). AGTR1 transactivation of MR may involve RAC1, which is highly activated in a mouse model of salt and angiotensin II excess (Fig. 1, section C). In this scenario, RAC1 inhibition reduces MR nuclear localisation and *SGK1* transcription to the same extent as eplerenone (Kawarazaki *et al*. 2012). Local production of aldosterone is not involved, as angiotensin II-treated VSMCs do not express aldosterone synthase and gene expression is not altered by aldosterone synthase inhibition (Jaffe & Mendelsohn 2005). Conversely, MR acts via AGTR1 to upregulate profibrotic markers such as collagen 1A (COL1A) and 3A (COL3A) and α-smooth muscle actin (SMA) (Tsai *et al*. 2013). Therefore, the MR and AGTR1 are intertwined at multiple points facilitating cooperation of different effector systems of RAAS with implications for both homeostasis and in disease states.

G-protein coupled oestrogen receptor (GPER, also known as GPER-1 or GPR30) As many cellular signalling cascades relay information from membrane surface to the interior, it was believed that a distinct membrane-bound MR exists. Radiolabelled binding assays showed mineralocorticoid binding to the plasma membrane of porcine renal cells and human monocytes with higher affinity than other steroids (Wehling *et al*. 1991, Christ *et al*. 1994). Furthermore, bovine serum albumin (BSA)-conjugated aldosterone triggers PKCα signalling (Le Moellic *et al*. 2004) and polyethylene glycol (PEG)-conjugated aldosterone activates ERK, despite both being too large to enter the cell to activate classical cytosolic MR (Ashton *et al*. 2015). Differential action of classical and alternative receptors is suggested by the latter study, where PEG–aldosterone could not upregulate classical MR target genes such as *SGK1*, yet unconjugated aldosterone could both upregulate *SGK1* and activate ERK. However, numerous experiments have failed to identify a unique membrane-bound MR. Instead, GPER is proposed as an alternative candidate for mineralocorticoid signalling.

GPER is a G-protein coupled receptor that is expressed in numerous tissues such as cardiomyocytes, VSMCs, vascular endothelium, lung, liver and reproductive tissues (Prossnitz *et al*. 2007, Jessup *et al*. 2010, Gros *et al*. 2011*b*). 17β-Oestradiol (E2) was the first known ligand for GPER, which is responsible for some of the rapid effects of E2 via MAPK (Filardo *et al*. 2000), and via PI3K signalling mediated by EGFR transactivation (Revankar *et al*. 2005). In GPER-transfected human embryonic kidney cells, which lack native oestrogen receptors, E2 exhibits rapid association/dissociation and high-affinity binding to the recombinant human GPER with a dissociation constant (K_d) of 2.7 nM (Thomas *et al*. 2005). In an *ex vivo* experiment, E2 concentrations of 0.1–10 nM are capable of inducing GPER-mediated changes to calcium handling in the renal connecting tubule (Hofmeister *et al*. 2012).

GPER may also be responsible for a subset of aldosterone's rapid cellular actions involving ERK signalling in rat aortic VSMCs (Gros *et al*. 2011*b*), endothelial cells (Gros *et al*. 2013) and rat H9C2 cardiomyocytes (Ashton *et al*. 2015). In these tissues, aldosterone activation of ERK could occur through either MR or GPER (Fig. 1, section B). Evidence for GPER signalling includes the maintenance of phosphorylation of ERK in rat endothelial tissue lacking MR (Gros *et al*. 2013), despite the eplerenone treatment in native GPER and MR expressing freshly isolated endothelium-denuded rat aorta (Gros *et al*. 2011*b*). Yet, ERK activation is inhibited

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with GPER antagonism or knockdown (Gros *et al*. 2011*b*, Ashton *et al*. 2015). Where both GPER and MR are co-expressed, the relative contribution to aldosterone-mediated ERK activation varies by cell type. In primary cultures of rat ventricular myocytes, GPER blockade inhibits ERK phosphorylation to a lesser degree than MR or AGTR1 antagonism, and does not affect MR-mediated ROS generation (Cannavo *et al*. 2016). Primary VSMC cultures tend to lose GPER expression over time, and in this context aldosterone can trigger ERK signalling via MR alone. However, when GPER is reintroduced through adenoviral transfection, MR predominantly acts through GPER (Gros *et al*. 2011*b*). There is ongoing debate as to whether aldosterone is a true ligand of GPER. Although there is apparent activation of GPER at physiological levels of aldosterone (e.g. 10 nM) in the above-mentioned studies, binding has not been definitively demonstrated (Cheng *et al*. 2014, Rigiracciolo *et al*. 2016). Alternative mechanisms of aldosterone action via GPER may include direct physical association between MR and GPER (Rigiracciolo *et al*. 2016), cross-talk via second messengers, GPER induction of local aldosterone synthase, and modification of the structural protein *striatin*, which can modulate steroid receptor function (Barton & Meyer 2015). However, the persistence of aldosterone responses in tissues lacking or deficient in MR and blocked by GPER antagonist is not explained by these alternative hypotheses (Feldman & Limbird 2015).

NOX, ROS and MR signalling

The MR activation of other membrane receptor signalling systems increases the diversity of its functions. These cross-talk interactions are necessarily context dependent to avoid non-specific activation. In particular, the redox status of cells is a major determinant of MR access to these alternative pathways. The generation of ROS is increased by MR activation, particularly through upregulation of NOX. NOX is a family of membrane-bound enzymes, which generate superoxide from NADPH and oxygen. NOX is present in leucocytes, where superoxide is required for the antimicrobial oxidative burst. It is also found in cardiomyocytes, endothelial cells and VSMCs (Ying 2008, Santillo *et al*. 2015). NOX-generated ROS has numerous regulatory functions including altering protein phosphorylation, enzymatic reactions, cellular ion transport, gene transcription, cell growth and death (Bedard & Krause 2007). In disease, enhanced NOX activity leads to excessive and dysfunctional activation of proinflammatory, profibrotic and angiogenic genes through the AP-1 and NF-κB pathways (Fiebeler *et al*. 2001, Queisser *et al*. 2011). Many subtypes of NOX exist, but in experimental RAAS overactivation, NOX2 is upregulated in heart tissue whereas NOX1 and NOX4 are not. This suggests specific isoforms are responsible for RAAS-induced cardiovascular oxidative stress and inflammation (Stas *et al*. 2007, Nakamura *et al*. 2009).

Aldosterone rapidly increases ROS generation by NOX within minutes in VSMCs (Callera *et al*. 2005) and cardiomyocytes (Hayashi *et al*. 2008, Tsai *et al*. 2010). The rapid onset of action and persistence of NOX generation of ROS, despite the inhibition of transcription and protein synthesis, strongly support a non-genomic mineralocorticoid contribution to regulation of NOX (Hayashi *et al*. 2008). The aldosterone effect is MR dependent in most studies (Callera *et al*. 2005, Hayashi *et al*. 2008, Iwashima *et al*. 2008), although one study using HL-1 atrial cardiomyocytes found no inhibitory effect of spironolactone (Tsai *et al*. 2010). MR activation of NOX is c-Src-dependent (Callera *et al*. 2005, Iwashima *et al*. 2008, Montezano *et al*. 2008, Cannavo *et al*. 2016), with downstream activation of RAC1 at least in endothelial cells. Here, activated MR increases GTP-bound RAC1 without increasing protein levels (Iwashima *et al*. 2008). RAC1 generates ROS by activating the NOX cytosolic subunit p47phox, which allows the assembly of other subunits into active NOX (Babior *et al*. 2002). Supplementing this process, MR activation also increases p47phox localisation to the cell membrane (Keidar *et al*. 2004, Miyata *et al*. 2005*a*, Nagata *et al*. 2006). However, there is a much slower increase in NOX activity over 6 h by aldosterone in endothelial cells suggesting that this process is distinct to that seen in VSMCs and cardiomyocytes (Iwashima *et al*. 2008). MR also signals via EGFR to increase NOX generation of ROS, and can synergise with angiotensin II to do so (Montezano *et al*. 2008). In cardiomyocytes, the MR-EGFR interaction utilises the PI3K/Akt cascade to activate NOX, which in turn triggers mitochondria to generate even more ROS in a feed-forward effect (Nolly *et al*. 2014). The MR–AGTR1 interaction separately contributes by inducing mitochondrial localisation of GRK2 which promotes ROS generation (Cannavo *et al*. 2016).

Additionally, MR upregulates NOX by genomic means: increasing synthesis of NOX cytosolic subunits in renal mesangial cells, endothelial cells and heart (Miyata *et al*. 2005*a*, Nagata *et al*. 2006, Stas *et al*. 2007). MAPK signalling remains important for NOX2 synthesis, as knockout of

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apoptosis signal-regulating kinase 1 (ASK1), a MAPK kinase kinase, attenuates aldosterone-induced cardiac NOX2 upregulation, superoxide generation and cardiac fibrosis (Nakamura *et al*. 2009). AGTR1 signalling is required for MR-mediated *Ncf1* transcription (the p47phox gene) in rat aorta, but not for other subunits (Hirono *et al*. 2007). This latter effect is in parallel to the AGTR1 synergy with MR in EGFR/PI3K signalling in cardiomyocytes discussed previously (Montezano *et al*. 2008).

Cellular redox status influences many of the cellular processes triggered by MR activation and even the method of MR activation itself (Fig. 1, sections B and C). For instance, ligand-free MR activation enabled in oxidative stress may partially explain the benefits to cardiac infarct healing with spironolactone treatment of adrenalectomised rats, despite the absence of endogenous ligands to activate the MR (Mihailidou *et al*. 2009). The generation of ROS is a necessary co-factor for certain MR signalling pathways; for example, antioxidant treatment attenuates the ability of aldosterone to transactivate EGFR (Huang *et al*. 2009) and IGF1R (Cascella *et al*. 2010). Also, some MR-mediated transcription could be redox sensitive including *SGK1*, *SLC9A1* (encoding for NHE-1), and some pro-inflammatory and profibrotic genes (Callera *et al*. 2005, Pinto *et al*. 2008, Nakamura *et al*. 2009). The specific mechanisms of ROS contribution to MR function and maladaptive organ remodelling and damage will be described in the next section.

Examples of coordinated MR transcriptional and rapid signalling effects in homeostasis and disease

Although most of the cell signalling systems activated by MR and mineralocorticoids are ubiquitous, a uniform coordinated response is observed within specific tissues. While this has been best characterised in renal tubular epithelial cells, there is expanding knowledge of the mechanisms of MR effect in the cardiovascular system and immune cells. In this section, the interaction between aldosterone, MR, second messenger systems, receptor transactivation and gene transcription will be illustrated in the context of organ function or disease.

Renal sodium handling

The MR is expressed in epithelial cells, most importantly in the distal nephron (Doucet & Katz 1981, Farman *et al*. 1982), but also in sweat glands (Kenouch *et al*. 1994), the gastrointestinal tract (Rafestin-Oblin *et al*. 1984) and mammary glands (Quirk *et al*. 1983), where it regulates cellular electrolyte handling. MR activation leads to both rapid and sustained homeostatic effects through a combination of second messenger signalling and early and later transcribed genes, which have been best characterised in the renal epithelial cell. This is illustrated in Fig. 2, and described in detail in the following sections.

MR regulation of target genes is the most potent determinant of its life-sustaining effects (Fig. 2, sections B and C). MR-knockout mice suffer early demise due to dehydration and salt wasting despite compensatory elevation in the components of the RAAS (Berger *et al*. 1998). This fate is shared by mice homozygous for a non-synonymous substitution in the MR DBD, which abolishes its ability to bind to DNA and regulate primary gene transcription (Cole *et al*. 2015). Hence, MR regulation of target genes is critical for this function. Examples of the effect of MR target genes are well described in renal physiology. All distal nephron epithelial cells express the ENaC, which is the major contributor to resorption of sodium in the distal nephron (Kellenberger & Schild 2002). ENaC is a heterotrimeric protein comprised of α-, β- and γ-subunits, which undergo intracellular processing before export via vesicles to the apical membrane where it becomes active (Eladari *et al*. 2012). MR activation increases sodium influx via ENaC, partially through direct transcription of the α-subunit (*Scnn1A*) (Masilamani *et al*. 1999, Mick *et al*. 2001). MR activation also increases total protein levels of the Na/K-ATPase pump within 24 h, which is responsible for exporting sodium out of the basolateral cell membrane to the interstitium (Alvarez de la Rosa *et al*. 2006).

MR also increases the expression of genes that regulate post-translational modifications of the electrolyte handling machinery, providing a more rapid response than direct synthesis of channels or transporters (Fig. 2, section B). *SGK1* is one such rapidly transcribed gene, which increases activity of ENaC (Chen *et al*. 1999). It also increases thiazide-sensitive sodium chloride cotransporter (NCC) activity (Faresse *et al*. 2012, Ko *et al*. 2013), which is a lesser contributor to renal sodium resorption (Gamba *et al*. 1994). The early effects of SGK1 are largely to preserve the active surface expression of ENaC and NCC. SGK1 phosphorylates the ubiquitin protein ligase Nedd4-2, preventing it from tagging ENaC or NCC for destruction (Snyder *et al*. 2002, Arroyo *et al*. 2011). SGK1 also directly interacts with the SCNN1A to increase the proportion of active open ENaC channels (Diakov & Korbmacher 2004), and promotes ENaC transcription (Zhang *et al*. 2007). With similar enhancing effects on NCC (Rozansky *et al*. 2009,

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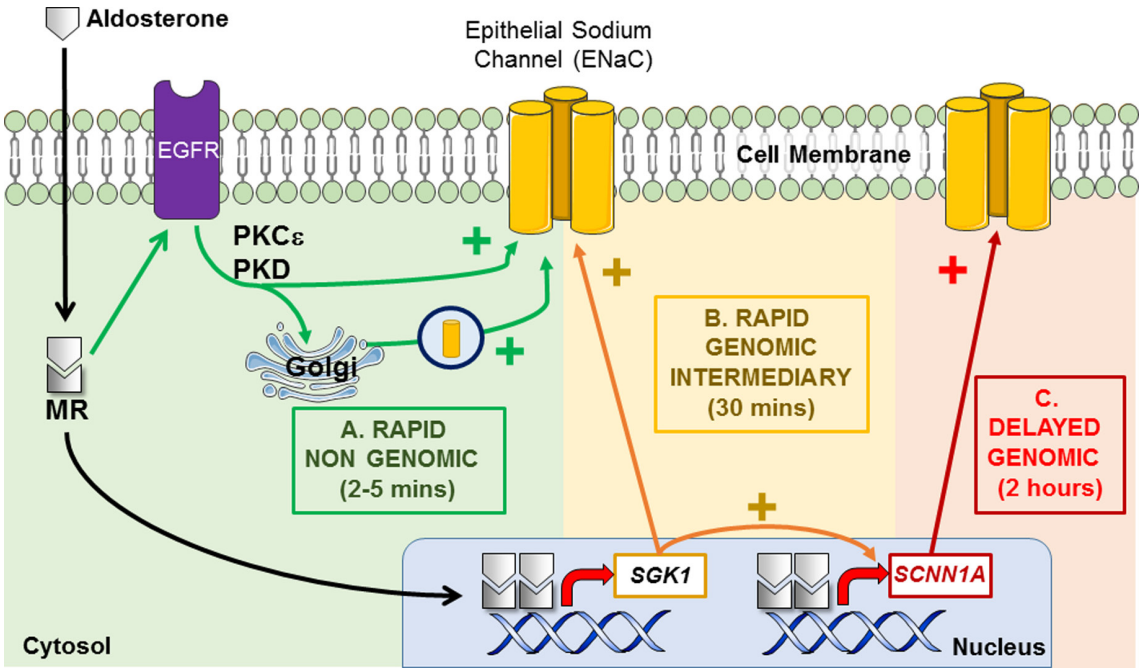

Figure 2
Time course of MR effects on epithelial sodium channel (ENaC) activity in renal epithelial cells. Once activated by aldosterone, the MR triggers rapid signalling pathways, and increases transcription of intermediary genes (such as *SGK1*, *CNKSR3* and *GILZ*) which enhance ENaC activity and prevent degradation, as well as direct transcription of *SCNN1A* which codes for the ENaC channel α-subunit. In combination, the MR can provide a rapid and sustained ENaC mediated sodium resorption in response to hypovolaemia.

Ko *et al*. 2013) and Na/K-ATPase expression and activity (Zecevic *et al*. 2004, Alvarez de la Rosa *et al*. 2006), SGK1 is crucial in early and delayed mechanisms of electrolyte transport. Other MR target genes act synergistically with *SGK1* to prevent ENaC and NCC destruction. Examples include ubiquitin-specific protease 2–45 (Oberfeld *et al*. 2011), CNKSR3 (Soundararajan *et al*. 2012) and GILZ1 (Soundararajan *et al*. 2010).

Non-canonical rapid MR-mediated effects on ENaC increase its surface expression and activity (Fig. 2, section A). MR signalling via IGF1R activates PI3K (Blazer-Yost *et al*. 1999), with products of PI3K directly interacting with ENaC to increase the probability of open channels (Pochynyuk *et al*. 2007). This generates a rapid but transient effect for 1 h, after which onset of genomic mechanisms (such as via *SGK1*) contribute to maintenance of ENaC activity (Holzman *et al*. 2007). Once *SGK1* is upregulated, PI3K also promotes SGK1 phosphorylation (Wang *et al*. 2001, Collins *et al*. 2003). MR transactivation of the EGFR, with downstream activation of PKC and PKD1, mediates aldosterone effects on ENaC subunit trafficking and membrane integration. PKCε is activated by aldosterone within 2 min, forming PKCε–PKD1 complexes and activating PKD1 within 5 min (McEneaney *et al*. 2007, 2008). Similarly, intracellular trafficking of ENaC subunits is enhanced within 2 min and ENaC subunit translocation from cytoplasm to cell membrane within 30 min (McEneaney *et al*. 2008, Dooley *et al*. 2013). ENaC subunits are initially packaged in the Golgi apparatus, emerging from the adjacent trans-golgi network in endosomes. Eventually these are directed towards, and insert into, the apical cellular membrane (Butterworth 2010). The MR-dependent increased activity of ENaC induced by aldosterone after 2–4 h is correlated with this redistribution, which cannot occur without PKD1 (McEneaney *et al*. 2008, 2010*b*, Dooley *et al*. 2013).

Second messenger systems activated by MR do not act in isolation, with components of some pathways capable of activating those of another. For example, K-RAS upregulates ENaC activity in a PI3K-dependent manner rather than via RAF-MEK-ERK1/2 (Staruschenko *et al*. 2004), which in fact is a negative regulator of ENaC (Grossmann *et al*. 2004). Occasionally, interactions between downstream second messengers can result in opposing cellular effects. For example, aldosterone induces PKD1 to rapidly form complexes with phosphatidylinositol 4-kinase IIIb (PI4KIIIb) in the trans-golgi network, which promotes export of ENaC subunits, and enhances the direct PKD1 effect on ENaC transport (Hausser *et al*. 2005, Dooley *et al*. 2013). However, PKD1 also prolongs MR-induced ERK1/2

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activation (McEneaney *et al.* 2010*a*), which increases degradation of ENaC via PKC, to mitigate the effect of increased ENaC expression (Booth & Stockand 2003).

Vasomotor and endothelial function

Vascular endothelial cells and VSMCs express the MR (Lombes *et al.* 1992), with MR signalling in these tissues contributing to regulation of vasomotor tone. However, the literature varies on if, and under what context, mineralocorticoids exert a constricting or relaxing effect, and whether that action is direct or via augmentation of responses to other vasoactive stimuli. A summary of MR signalling in vascular function is presented in Fig. 3.

Endothelial MR influences NO levels, which impacts on vascular tone (Fig. 3, top section). NO is generated by eNOS, which diffuses into adjacent VSMCs, and triggers generation of cyclic guanosine monophosphate (cGMP) which ultimately results in relaxation (Förstermann & Münzel 2006). In bovine aortic endothelial cells, MR rapidly signals via PI3K/Akt to increase eNOS production of NO within 2 minutes (Liu *et al.* 2003, Mutoh *et al.* 2008). However, MR activation reduces eNOS activity in HUVECs (Hashikabe *et al.* 2006). Rapid induction of RhoA kinase activity by MR maximally reduces eNOS activity within 15 min by inhibition of Akt (Kirsch *et al.* 2013), while prolonged MR activation (16 h) also inhibits eNOS activity by increasing protein phosphatase 2A activity, which dephosphorylates eNOS (Nagata *et al.* 2006). As MR acts through different pathways with opposing outcomes, context is important in determining its effect on eNOS.

Vascular endothelial MR genomic effects increase oxidative stress. The NOX subunit p47phox has increased expression and membrane localisation in response to MR activation, with ROS generation after 2 h (Nagata *et al.* 2006). Additionally, aortic expression of cyclooxygenase (COX)-2 is increased in aldosterone-treated rats (Blanco-Rivero *et al.* 2005, Eatman *et al.* 2011). COX-2 generates vasoactive prostanoids and ROS (Félétou *et al.* 2011), which impairs vasodilatory (Blanco-Rivero *et al.* 2005) and

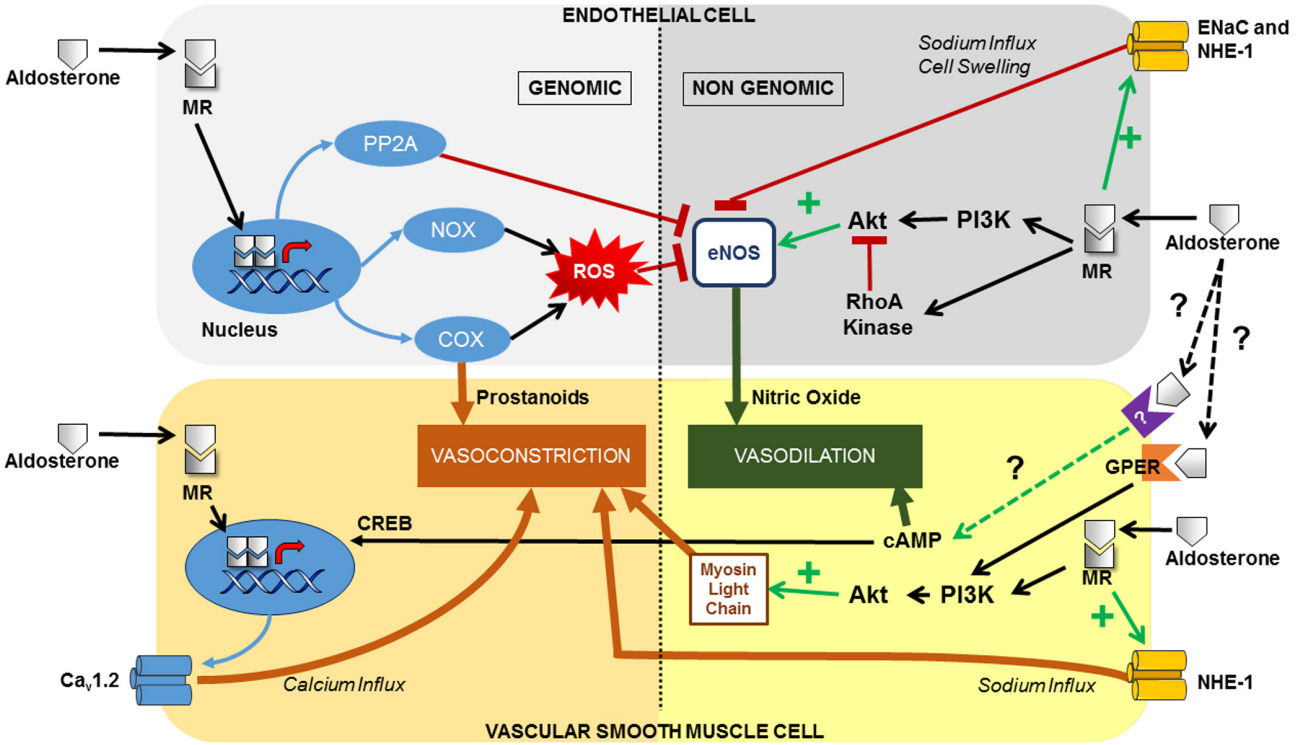

Figure 3
Mineralocorticoid mediated genomic (left) and non-genomic (right) events in the control of vascular function. In both endothelial (top section) and vascular smooth muscle cells (VSMCs), the PI3K pathway is an important second messenger system through which MR activates endothelial nitric oxide synthase (eNOS) and VSMC contractile elements such as myosin light chain (MLC). In VSMC, the presence of GPER enhances the effect through unknown mechanisms. Rapid effects on the ENaC sodium channel and sodium/hydrogen exchanger (NHE-1) are pro-constrictive. In VSMC, rapid increases to cAMP leads to upregulation of the CREB transcription factor, linking rapid signalling and genomic transcription. Genomic effects in endothelium includes increased synthesis of protein phosphatase 2A (PP2A) which deactivates eNOS, and NADPH oxidase (NOX) and cyclooxygenase (COX) which generate reactive oxygen species (ROS) impairing eNOS function. In VSMC, the MR can upregulate $Ca_v1.2$, an L-type voltage dependent calcium channel. The balance between different MR effects and other *in vivo* mediators determines overall vasomotor tone.

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enhances vasoconstrictive responses (Eatman *et al*. 2011). The effect of aldosterone on COX-2 expression is not uniform; it induces upregulation of COX-2 in the aorta and renal arteries, whereas it induces downregulation in the femoral artery (Eatman *et al*. 2011). These studies did not specifically investigate if these aldosterone effects were MR mediated, although in two different studies, eplerenone mitigated both angiotensin II (Rocha *et al*. 2002*a*) and aldosterone (Rocha *et al*. 2002*b*) induced cardiac COX-2 upregulation in rats. MR activation reduces glucose-6-phosphate dehydrogenase (G6PD) expression, which worsens oxidative stress and impairs both NO generation and NO-dependent vasodilation (Leopold *et al*. 2007). This environment of MR-mediated oxidative stress can compound adverse events such as the inactivating oxidative modification of endothelin-B receptor, which prevents its stimulation of eNOS (Maron *et al*. 2012). It also contributes to depletion of tetrahydro-L-biopterin (BH4), a potent reducing agent and co-factor for NO generation by eNOS. Depletion of BH4 uncouples eNOS, causing production of more ROS instead of NO, and is a contributing mechanism for MR-mediated reduction in endothelial NO production (Förstermann & Münzel 2006, Nagata *et al*. 2006, Chen *et al*. 2016). In PA, BH4 depletion and oxidative stress correlates with impaired endothelial healing in response to injury (Chen *et al*. 2016).

Aldosterone and MR also exert NO-independent effects on vasomotor function. Endothelial cell volume and tension are increased by aldosterone with deleterious effect. Aldosterone-induced rapid activation of NHE-1 (Schneider *et al*. 1997) and/or ENaC (Oberleithner *et al*. 2003) contributes to swelling, which is transient. Later, MR-mediated synthesis of cytosol-crowding macromolecules occurs with prolonged aldosterone exposure, which stiffens the cell and renders it susceptible to shear stress (Oberleithner *et al*. 2006). MR antagonism blocks all but the very early (<1 min) changes to cell volume and stiffness (Oberleithner *et al*. 2006).

Activation of the endothelial MR can both increase and reduce vascular reactivity and tone suggesting a complex regulatory framework, but there is also heterogeneity of experimental conditions in the literature. These differences include anatomical site, steroid dose and duration of exposure, and environmental context. For example, endothelial cell-specific MR deletion improved NO-dependent vasodilatory responsiveness after 2 weeks of angiotensin II exposure in mesenteric but not coronary arterioles (Mueller *et al*. 2015). Also, in bovine aortic endothelial cells, eNOS activity is maximally activated by picomolar to nanomolar concentrations of aldosterone, with diminished effect at higher concentrations (Liu *et al*. 2003, Leopold *et al*. 2007). However, in human coronary microarteries, maximal eNOS activation required higher than micromolar concentrations (Batenburg *et al*. 2012). Furthermore, while early rapid MR effects in afferent renal arterioles promote vasodilation, delayed-onset genomic effects can be vasoconstrictive (Uhrenholt *et al*. 2003). The heterogeneity extends to signalling through other receptors. Aldosterone activation of GPER results in an endothelium-dependent vasodilatory tendency in rat aorta (Gros *et al*. 2013), but potentiates angiotensin-II-induced vasoconstriction in human coronary arteries in an MAPK- and NO-independent mechanism (Batenburg *et al*. 2012). As AGTR1a signalling is important for aldosterone-mediated endothelial dysfunction (Briet *et al*. 2016), this discordance may reflect a specific AGTR1 effect in the latter study. *In vitro* cell culture and *ex vivo* isolated vessel experimental systems cannot replicate the complex *in vivo* milieu of changeable and interacting autonomic, endocrine, paracrine and stress related inputs, which together generate more unity of purpose than seen across individual experiments.

In the VSMC, MR signalling is important for maintaining basal tone and vascular contractile responses (Fig. 3, bottom section). If MR is deleted, cGMP- and calcium-dependent signalling is impaired with reduction in baseline activation of the contractile regulators myosin light chain kinase (MLCK) and myosin light chain (MLC) 2 (Tarjus *et al*. 2015*a*). The phosphorylation of MLC by MLCK is a necessary step in enabling actin–myosin coupling (Goulopoulou & Webb 2014) and occurs within 15 min of MR activation via PI3K signalling (Gros *et al*. 2011*a*). The basal expression of genes coding for contractile elements, ion channels or signalling systems is unaffected by MR deletion in VSMC (Tarjus *et al*. 2015*a*). However, MR does regulate $Ca_v1.2$ gene expression in mesenteric artery VSMC, an L-type calcium channel which increases vasomotor tone when active (McCurley *et al*. 2012). Aldosterone can also act in an MR-independent mechanism to increase VSMC cAMP levels within 1 min, which activates the transcription factor CREB within 10 min, linking rapid signalling with genomic transcription (Christ *et al*. 1999).

Sodium handling in VSMCs is under mineralocorticoid control. NHE-1 activity and sodium influx are increased by aldosterone in a biphasic manner: a rapid MR-independent mechanism and a prolonged MR-dependent response (Miyata *et al*. 2005*b*, Carreno *et al*. 2015). The resultant rise in intracellular sodium is exacerbated by an early transient MR-induced,

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PKC-dependent reduction in VSMC Na/K-ATPase surface activity and expression (Alzamora *et al*. 2003). However, with sustained MR activation and sodium influx, there is increased Na/K-ATPase subunit transcription (Muto *et al*. 1996). These changes affect cellular membrane potentials and calcium handling, with potential consequences on VSMC function (MR-mediated NHE-1 activity contributes to vasoconstrictive responses in the aorta) (Carreno *et al*. 2015).

Functionally, VSMC MR generally promotes a contractile response, augmenting the constrictor effect of thromboxane-A2 and angiotensin II in aged animals (Gros *et al*. 2011*a*, McCurley *et al*. 2012). VSMC MR has a role in hypertension, with VSMC MR knockout mice having lower basal blood pressures (Galmiche *et al*. 2014) and protection against age-related increases to systolic blood pressure (McCurley *et al*. 2012). However, MR also is important for NO-mediated relaxation of VSMC, and increases cAMP that has a vasodilatory effect, which may be autoregulatory in the presence of functional endothelium (Christ *et al*. 1999, Tarjus *et al*. 2015*a*).

The cardiac action potential, excitation–contraction coupling and electrical remodelling

The identification of MR expression in human cardiomyocytes indicates that MR exerts direct effects on the heart (Bonvalet *et al*. 1995, Lombes *et al*. 1995). Cardiomyocyte contraction is critically dependent upon intracellular calcium, which binds to troponin-C, unleashing a cascade of events that eventually facilitate actin and myosin filament movement and sarcomere contraction. Calcium influx and release from the sarcoplasmic reticulum is triggered by electrical depolarisation of the vesicle membrane. Atrial and ventricular cardiomyocytes are prone to rapid depolarisation, with their electrical status determined by the actions of sodium, calcium and potassium channels. Voltage-gated calcium channels are important for coupling depolarisation to contraction, by facilitating calcium influx into the cell and triggering the release of calcium from the sarcoplasmic reticulum (Lipscombe 2002). MR activity can thus modulate cardiomyocyte electrolyte handling, the action potential and cardiac contractility.

Cardiomyocytes express both low-voltage-activated T-type channels which exhibit rapid activation and slow deactivation, and L-type dihydropyridine channels which activate more slowly but deactivate more rapidly than T-type channels (Lipscombe 2002). Both are also important for pacemaker activity and propagation of action potentials. MR activation increases calcium current through both L-type and T-type calcium channels (Lalevee *et al*. 2005, Boixel *et al*. 2006). The calcium status of cardiomyocytes is strongly linked to transmembrane sodium concentrations (Bogeholz *et al*. 2012, Aronsen *et al*. 2013). Aldosterone raises intracellular sodium levels by rapidly promoting sodium influx through the NHE-1 (Korichneva *et al*. 1995, Matsui *et al*. 2007) within 10 min via MR transactivation of EGFR (De Giusti *et al*. 2011), the electrogenic sodium/bicarbonate cotransporter (SLC4A4) via GPER and PI3K/Akt (De Giusti *et al*. 2015, Orlowski *et al*. 2016), and the Na-K-2Cl cotransporter (SLC12A) via a PKCε-dependent pathway (Mihailidou *et al*. 1998, 2004). This PKCε pathway also mediates a reduction in Na/K-ATPase activity, inhibiting sodium export (Mihailidou *et al*. 2000, 2004). While SLC12A activity continues with prolonged MR signalling, the Na/K-ATPase inhibition is only transient (Mihailidou *et al*. 2004).

As a regulator of intracellular pH and cell volume through the exchange of sodium for hydrogen, NHE-1-dependent cellular alkalinisation can increase myofilament responsiveness to calcium (Mattiazzi 1997) and may explain aldosterone's rapid inotropic effect on cardiomyocytes (Barbato *et al*. 2002, 2004). NHE-1 is also important in generating a stretch-induced secondary slow force contractile reaction that occurs after the initial Frank–Starling response. It is postulated that an angiotensin-II-mediated local generation of aldosterone acts via EGFR, with downstream ROS generation and ERK1/2 phosphorylation activating NHE-1 to trigger contraction. MR knockdown with hairpin interfering RNA blocks the slow force response, with reduced ERK1/2 phosphorylation and NHE-1 activity (Diaz *et al*. 2014). Although this hypothesis is controversial due to difficulty in identifying aldosterone synthase in the heart (Ye *et al*. 2005), increased aldosterone synthase gene expression in heart failure patients (Yoshimura *et al*. 2002) and the persistence of aldosterone in the hearts of adrenalectomised rats (Gomez-Sanchez *et al*. 2004) suggest some cardiac capacity for aldosterone generation in response to major disturbances to normal function.

There is the possibility that MR can mediate electrical dysfunction. Cardiac-specific overexpression of MR in mice leads to an increase in action potential duration and ventricular arrhythmia due to aberrant release of calcium from the sarcoplasmic reticulum (Ouvrard-Pascaud *et al*. 2005, Gomez *et al*. 2009). In humans, PA patients are at higher risk of atrial fibrillation (AF) compared to age and blood pressure matched controls (Milliez *et al*. 2005,

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Catena *et al*. 2008, Savard *et al*. 2013). Electrical remodelling, such as upregulation of calcium channels and downregulation of potassium channels, precedes MR-mediated structural remodelling suggesting a dual mechanism for arrhythmia pathogenesis (Lalevee *et al*. 2005, Ouvrard-Pascaud *et al*. 2005).

Cardiovascular inflammation, fibrosis and repair

Chronic excessive MR activation is uniformly associated with adverse cardiovascular outcomes, as seen in PA. The persistent excessive secretion of aldosterone is associated with hypertension and end organ disease, including cardiac left ventricular hypertrophy (Rossi *et al*. 1996, 2013) and renal impairment (Sechi *et al*. 2006). Patients with PA have increased risk of significant cardiovascular disease (CVD) events, such as stroke and myocardial infarction (MI), beyond that attributable solely to hypertension (Milliez *et al*. 2005, Mulatero *et al*. 2013). Treatment reduces the risk of significant CVD events to that experienced by treated primary ('essential') hypertension patients (Catena *et al*. 2008).

Curiously, there is clinical evidence of benefit when MR antagonists are used in disease states unrelated to mineralocorticoid excess, such as heart failure after myocardial infarction (Pitt *et al*. 2003). In animal models of cardiac damage from pressure overload (Lother *et al*. 2011, Li *et al*. 2014), oxidative stress (Usher *et al*. 2010, Bienvenu *et al*. 2012, Coelho-Filho *et al*. 2014), valvular incompetence (Zendaoui *et al*. 2012) and MI (Delyani *et al*. 2001, Enomoto *et al*. 2005, Takeda *et al*. 2007). Cardiac remodelling with impairment to systolic and/or diastolic function is attenuated through either cell-specific MR knockdown/deletion, or use of MR antagonists such as spironolactone and eplerenone. These benefits may be due to inhibition of glucocorticoid rather than mineralocorticoid activation of MR. However, the presence of endogenous ligands may not be required for MR-mediated adverse outcomes, with ongoing protection from spironolactone in an animal model of MI after adrenalectomy (Mihailidou *et al*. 2009) and the potential for RAC1-induced ligand-free MR activation (Nagase *et al*. 2012). This suggests that MR signalling influences cardiovascular recovery from injury through multiple mechanisms, and the contribution of MR to pathology extends more broadly than hyperaldosteronism or hypertension.

Persistent MR overactivation is associated with perivascular and cardiac inflammation within 14 days of constant mineralocorticoid exposure (Rocha *et al*. 2002*b*, Usher *et al*. 2010, Rickard *et al*. 2012). This arises after upregulation of factors that enhance leucocyte recruitment, adhesion and infiltration. In endothelial cells, this includes intercellular adhesion molecule (ICAM1), CCR5 and P-selectin (Caprio *et al*. 2008, Jeong *et al*. 2009, Rickard *et al*. 2014). Also, MR induces placental growth factor production in VSMC, which recruits monocytes via FLT1, a vascular endothelial growth factor (VEGF) receptor (McGraw *et al*. 2013). MR can indirectly regulate transcription of genes involved in recruitment and adhesion, with ICAM1 and vascular cell adhesion molecule-1 (VCAM1) protein expression upregulation via PDGFR and c-Src activation (Callera *et al*. 2011*a*), and osteopontin via ERK and p38 MAPK (Fu *et al*. 2012). Once recruited, MR signalling is important for activating and influencing the behaviour of inflammatory cells. Myeloid cells increase the generation of pro-inflammatory cytokines such as TNF-α, IL-1b and IL-6 in response to aldosterone (Usher *et al*. 2010). Many of these are under the regulation of NF-κB, whose activity is enhanced by SGK1 (Zhang *et al*. 2005, Leroy *et al*. 2009, Ding *et al*. 2012). Conversely, macrophages derived from peripheral blood monocytes of healthy human volunteers developed an anti-inflammatory, pro-healing genetic transcription profile in response to MR antagonist treatment. This profile is similar to that induced by IL-4, which is known to polarise macrophages to an anti-inflammatory phenotype (Labuzek *et al*. 2013).

Matrix metalloproteinases (MMPs) degrade collagen and cleave precursors of pro-inflammatory cytokines into active forms (Schonbeck *et al*. 1998). MR activation upregulates MMP production utilising various second messenger pathways, enhancing inflammatory cell infiltration. In neutrophils, increased transcription of *MMP9* and pro-angiogenic *VEGFA* by MR requires intact PI3K, p38 MAPK and ERK signalling (Walczak *et al*. 2011, Gilet *et al*. 2015). In myeloid cells, such as macrophages, MMP12 production requires intact MR signalling via JNK/AP-1 and ERK cascades (Shen *et al*. 2016). In cardiomyocytes, PKC and the generation of ROS by NOX are prerequisites for MR-mediated ERK activation and MMP9 generation (Rude *et al*. 2005). The MR-induced ROS oxidises calcium/calmodulin-dependent protein kinase II (CAMK2), which drives Mmp9 transcription by myocyte enhancer factor 2 (MEF2) (He *et al*. 2011).

A number of MR-regulated genes are mitogenic, pro-hypertrophic and profibrotic and, similar to chemoattractant factors, are subject to indirect MR

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regulation using second messenger systems. For example, MR acts through ERK signalling to induce cardiac fibroblast proliferation (Stockand & Meszaros 2003) and cardiomyocyte transcription of hypertrophy-associated proteins such as α- and β-myosin heavy chain (Okoshi *et al*. 2004). Additionally, MR signalling via p38 MAPK promotes cardiomyocyte production of connective tissue growth factor (CTGF), which is a profibrotic stimulus (Lee *et al*. 2004). Transactivation of other receptor systems is involved in MR-mediated remodelling. AGTR1 transactivation is required for the upregulation of fibrotic and hypertrophic genes such as transforming growth factor-beta (TGF-β), *Col1a*, *Col3a* and *Acta2* (which encodes α-smooth muscle actin) in cardiomyocytes via ERK and JNK (Tsai *et al*. 2013), while the pro-hypertrophic MEF2 requires AGTR1 signalling via the G-protein coupled receptor kinase (GRK) 5 (Cannavo *et al*. 2016). In cardiac fibroblasts, aldosterone acts via an unknown membrane G-protein coupled receptor (and not MR) to transactivate IGF1R via c-Src, with downstream PI3K/Akt signalling leading to elastin production (Bunda *et al*. 2009). EGFR transactivation by cardiac MR increases NHE-1 activity (Fujisawa *et al*. 2003, Young & Funder 2003, De Giusti *et al*. 2011) with resultant sodium accumulation promoting calcium influx and activation of MAPK (p38, ERK), Akt, calcineurin and CAMK2. This facilitates the generation of pro-hypertrophic factors (Darmellah *et al*. 2007, Nakamura *et al*. 2008). EGFR transactivation may be profibrotic, as it is mitogenic in a renal fibroblast cell line via JNK, ERK and PI3K/Akt cascades (Huang *et al*. 2012). However, *in vivo* impact on fibrosis may be limited, with impaired EGFR function not protecting mice against mineralocorticoid-induced cardiac remodelling (Messaoudi *et al*. 2012). Several profibrotic genes are directly regulated by MR as a transcription factor. As in the kidney, MR increases *SGK1* transcription in the heart (Martin-Fernandez *et al*. 2011). SGK1 upregulates the profibrotic CTGF (Vallon *et al*. 2006, Terada *et al*. 2012), and the importance of *SGK1* in the pathogenesis of MR-mediated cardiac fibrosis has been established in a knockout mouse model (Vallon *et al*. 2006). Neutrophil gelatinase-associated lipocalin (*Lcn2)* is a directly MR-regulated gene in cardiomyocytes (Latouche *et al*. 2012). LCN2 is a stimulus for fibroblasts to deposit type 1 collagen and plays a pathological role in MR-mediated coronary perivascular fibrosis (Tarjus *et al*. 2015*b*).

Oxidative stress is a key facilitator of adverse remodelling and inflammatory effects of the MR including rapid effects on vascular function and the increased transcription of culprit genes. As MR activation simultaneously promotes production of ROS, particularly through NOX2, a self-sustaining interaction could exacerbate and potentiate inflammation and fibrosis. MR is important for the transcription of NOX and its p22phox subunit (Fiore *et al*. 2009). In the heart, this appears to be driven by infiltrating macrophages, as prevention of their recruitment reduces NOX upregulation and cardiac fibrosis (Rickard *et al*. 2012, Shen *et al*. 2014). Aldosterone acts via MAPK to increase NOX2, CCL2 and TGF-β1 expression and to cause cardiac fibrosis (Nakamura *et al*. 2009). NOX-generated ROS also contributes to vascular remodelling. MR-induced IGF1R expression, activation and downstream signalling (via MAPK and PI3K/Akt) with subsequent VSMC cell proliferation and migration are ROS dependent (Cascella *et al*. 2010). Similarly, the reparative function of endothelial progenitor cells from PA patients is impaired by eNOS uncoupling related to increased NOX production of ROS (Chen *et al*. 2016). Therefore, redox status determines the outcome of several MR-mediated pathological processes.

Conclusions

So far, the uncovered mechanisms of action of mineralocorticoids and the MR paint a picture of a sophisticated multifunctional system. Harnessing cellular second messenger systems while genomic transcription events are given sufficient time to increase and sustain its defence against hypovolaemia, MR activation in the kidney and vessels shows itself to be an agile and powerful preserver of homeostasis. Yet, MR activation and triggering of the same genes and signalling pathways elsewhere and under different circumstances can lead to recruitment of inflammatory cells and fibrosis or maladaptive repair in response to injury. There is an increasing body of work regarding the contribution of various MR expressing cell types to tissue inflammation, fibrosis, maladaptation and hypertension, but there is a concurrent need to map the gene targets and intracellular signalling pathways underlying these outcomes. Eventually, this could lead to novel therapeutic options such as targeting transactivated receptors and superoxide generation in combination with MR antagonism. There is also scope for development of new agents that preferentially obstruct pathological signalling whilst preserving the essential electrolyte regulatory effects of MR.

There are many unresolved issues in mineralocorticoid and MR signalling. It is likely that

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additional mechanisms protect the MR against non-specific activation by its several high-affinity ligands or context-dependent ligand-free activation. Similarly, there is more to discover about the membrane receptors through which mineralocorticoids can induce effects without binding to its classical MR, although there is increasing evidence that GPER is involved. The mechanism of MR interaction with GPER itself is incomplete, and other candidate receptors may exist including the elusive membrane bound MR. However, current research methods in this area largely rely on *in vitro* and *ex vivo* experiments in isolated systems, which cannot account for the numerous contributing inputs in *in vivo* systems. As the body of research expands, there is a risk of confusion from inconsistencies and variations in mechanisms and functional outcomes between studies. Instead, we hope that clearer patterns will emerge, leading us closer to the intelligent design behind the multifunctional MR.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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