Aldosterone-Mediated Renal Sodium Transport Requires Intact Mineralocorticoid Receptor DNA-Binding in the Mouse

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The classic role of mineralocorticoid receptor (MR) is to promote sodium transport in epithelial tissues. However, the MR is also expressed in a range of tissues in which its role appears unrelated to sodium transport, and under normal physiological conditions, it may be responding to cortisol (corticosterone in rodents) rather than aldosterone. The relative importance of transcriptional mechanisms such as classical genomic signaling via a hormone response element, transrepression of other transcription factors, and nongenomic signaling is not clear, particularly in nonepithelial tissues. The goal of the present study was to define the role of the different signaling pathways for the MR by separating the functional role of classic genomic signaling, mediated by DNA binding, from these two other mechanisms. We used gene targeting to generate mice in which serine is substituted for cysteine at codon 603 in the MR; this mutation precludes DNA binding. These MR C603S mutant mice either die at birth or fail to thrive, lose weight, and die between days 10 and 13 in a manner similar to that observed previously for mice null for the MR gene. Renal expression and cellular localization of MR C603S by immunohistochemistry was equivalent to control mice. MR C603S mice were rescued by twice-daily saline injections. Despite increased aldosterone levels, renal expression of aldosterone-induced genes was not increased. This unique mouse model demonstrates that DNA binding is essential for the epithelial MR response and will provide the basis for analysis of nonclassical signaling of the MR in nonepithelial tissues. (Endocrinology 156: 2958–2968, 2015)

The mineralocorticoid receptor (MR) plays a central role in the maintenance of sodium homeostasis by mediating aldosterone-induced vectorial sodium transport in the distal nephron and distal colon (1). Genetic deletion of the MR in transgenic mice (2) or haplo-insufficiency in the inherited disease pseudohypoaldosteronism type 1 (3) is associated with profound neonatal salt wasting. Mice with homozygous null mutations for the MR fail to thrive and generally die of dehydration at approximately day 10 (2).

The MR is, however, expressed in a range of tissues, only some of which relate to sodium transport; indeed, in many of these tissues, the physiological ligand is likely to be cortisol (corticosterone in rodents) (1). The MR has at least two physiological ligands, aldosterone and cortisol, with ligand specificity at the tissue level being achieved by the enzyme 11β-hydroxysteroid dehydrogenase type II (4). Thus, in tissues including the brain (5), macrophages (6), the cardiovascular system (7), and adipose tissue (8), the MR is expressed in the absence of 11β-hydroxysteroid dehydrogenase type II and by implication is a receptor for cortisol (9).

The MR is a member of the nuclear receptor superfamily of ligand-activated transcription factors. These receptors have three principal functional domains. The N-ter-
mineral domain is relatively unstructured, particularly in the MR (10), allowing a diversity of interactions, including an interaction with the ligand-binding domain (LBD) (11). The central DNA-binding domain (DBD) is highly conserved across all members of the nuclear receptor superfamily (12), whereas the LBD has a well-conserved tertiary structure (13–15). The LBD shares close sequence homology with the other members of the steroid receptor subfamily, particularly the glucocorticoid (GR), progesterone, and androgen receptors; this homology is reflected in the pattern of ligand binding in that agonist and antagonist ligands may bind more than one of these receptors, albeit with varying affinities. This ligand discrimination is imposed by the region between helices 5 and 6 in the C-terminal LBD (16).

Steroid receptors transduce their signaling in cells through three basic mechanisms: in the classical genomic pathway, the receptor acts in trans by interacting with a cis-element, the steroid response element, in a regulated gene (17). This interaction is mediated by the DBD with the transactivation mediated by activating functions in both the N terminus (activation function-1) and the LBD (activation function-2), which interact with coregulatory molecular complexes (18, 19) and the transcriptional apparatus.

Transrepression is also a genomic mechanism but in this case involves a protein-protein interaction with other transcription factors. The best-characterized example is between the GR and the transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NFκB), which contributes to the antiinflammatory effect of glucocorticoids (20–22). In this mechanism, the GR does not bind directly to a hormone response element (HRE). In contrast to the GR, transrepression by the MR has not been clearly established. The MR does not interact with AP-1 (20), and a previously reported interaction with NFκB subunit in vitro (23, 24) is not consistent with results from other studies (25–28). NFκB activation stimulates expression of the adhesion molecule, intercellular adhesion molecule-1 (29) as does MR activation (30), whereas the GR transrepresses these responses (29). It is also counterintuitive, given the large body of work that demonstrates the effects of MR activation to be proinflammatory in the cardiovascular system and kidney (7), including activation of the transcription factors. The best-characterized example is a previously reported interaction with NFκB established. The MR does not interact with AP-1 (20), and to the GR, transrepression by the MR has not been clearly defined. In contrast, the GR does not bind directly to a hormone response element (HRE). In this mechanism, the MR acting at the cell membrane can activate second-messenger pathways, initiating so-called rapid or nongenomic signaling (34, 35).

Insights into the relative contributions to steroid hormone action of HRE-dependent and HRE-independent (transrepression and nongenomic) mechanisms have been obtained for the GR (36), ER (37), and androgen receptor (38) from studies in transgenic mice in which the ability of the steroid receptor to bind DNA has been compromised by targeted mutation. This approach has not previously been applied to the MR despite both the importance of the MR as a therapeutic target in cardiovascular disease and despite the limitations of in vitro system for analysis of the mechanisms of aldosterone-mediated signaling (39).

We have used the well-validated DBD mutation in which a serine is substituted for the cysteine at amino acid 603 in the mouse MR (40). The C603S mutation was created by using gene targeting to introduce this mutation into exon 3 of the mouse MR gene. The mice have been successfully bred to homogeneity. The mice exhibit profound salt-wasting essentially equivalent to that previously reported with the MR knockout mice, leading us to conclude that, at least with respect to epithelial sodium transport, binding of the MR to its hormone response elements is integral to function with the mutant MR unable to compensate through alternate signaling mechanisms.

### Materials and Methods

**Gene targeting a knock-in mutation in exon 6 of the mouse MR gene**

The MR C603S mice were created at the Monash Gene Targeting Facility (Monash University) by homologous recombination in C57BL/6 ES cells using a positive-negative selection approach in embryonic stem (ES) cells. A schematic showing the position of the mutation in exon 3 of the MR gene and the targeting strategy is shown in Figure 1. A bacterial artificial chromosome containing the mouse MR gene was isolated and a single amino acid substitution of a Cys to a Ser in position 603 (codon TGC was changed to AGC) of the mouse MR coding region was introduced by RecET (recombination ET system)-mediated recombination in Escherichia coli. A neomycin resistance selection cassette flanked by FLP recombinase target (FRT) recombination binding sites was inserted approximately 300 bp downstream of exon 6. The AscI linearized targeting vector was electroporated into ES cells and 400 G418-resistant colonies picked after 7 days of cell culture. Purified genomic DNA from ES cell...
clones were analyzed by Southern blot analysis using both single-copy 5'- and 3'-specific probes (Figure 1C). Ten correctly targeted ES cell clones were identified by Southern analysis, and three were injected into Balb/c host blastocysts to derive chimeric mice. Male chimeras from all three clones transmitted the targeted allele to their offspring to generate MRC603S heterozygous mice. Male and female heterozygotes were then crossed to generate mice homozygous for the C603S mutation. To exclude the possibility that the observed phenotype was in part due to the neomycin resistance gene cassette in the MR gene locus, a second neomycin resistance gene cassette in the MR gene locus, a second neomycin cassette to be excised from the genome. A 93-bp FRT flanking site sequence was left behind at the locus, which was used to distinguish between genotypes using standard PCR of mouse tail DNA.

**In vitro analyses of the MR C603S mutation**

Details of the Western blot analysis, ligand-binding assay, and transactivation assay are provided in the Supplemental Material.

**Rescue of MR C603S mice**

Mice were rescued by sc saline (0.9% NaCl in H2O) injection of approximately 30 µL/g body weight twice daily from postnatal day 5 using a Novo Pen 3 (Novo Nordisk Copenhagen) with Novo Fine 32-gauge needles until day 21 when they received saline to drink ad libitum and their food pellets were soaked in normal saline. All procedures with mice were approved by the Monash University Animal Ethics and Biosafety Committees (MMCB 2011/29).

**Genotyping**

Tail tips were obtained between days 7 and 8 for DNA extraction as previously described (6). For the strain containing the neomycin resistance gene cassette, three primers (forward: 5'-TGCAAGTTCCAGTTTCACGAG; Neo: 5'-GGTTGATATGGAATGTG TGC; reverse: 5'-TGGCATCTCGTTTCCACACG) were used, whereas for the second strain lacking the neomycin cassette, only primers forward and reverse were needed. Tail genomic DNA was subject to PCR using GoTaq DNA polymerase (Promega) with denaturation at 94°C for 5 minutes, initially and thereafter for 1 minute with annealing at 60°C for 1 minute and extension at 72°C for 1 minute for 35 cycles. The PCR products were subjected to electrophoresis in a 1.2% agarose gel (shown in Figure 1D).

**Tissue collection**

Mice were killed by CO2/O2 asphyxiation at 8 days or 5–6 weeks, and this was followed by the collection of arterial blood, the kidney, heart, and aorta. The heart and aorta were immediately halved and half fixed in 4% paraformaldehyde for histology and the apex snap frozen in liquid nitrogen for RNA extraction and analysis by quantitative RT-PCR.

**RIA for serum hormone levels**

The plasma concentrations of aldosterone, corticosterone, and angiotensin II were determined using ImmunChem double-antibody RIA kits (MP Biomedicals) as per the manufacturer’s instructions (6).

**Histological and immunohistochemical analyses**

Paraformaldehyde-fixed, paraffin-embedded kidney sections (5 µM) were stained for histology with either periodic acid-Schiff stains (Sigma-Aldrich) or hematoxylin and eosin stain. Immunohistochemical staining for the MR was performed on dewaxed and rehydrated kidney sections (5 µM thick) after citrate boiling antigen retrieval. The MR2B7 mouse monoclonal antibody for the MR (1:20; a kind gift from Professor Celso Gomez-Sanchez, University of Mississippi, Oxford Mississippi) using the animal research peroxidase kit for mouse primary antibodies (Dako) as per the manufacturer’s instructions (42).

**Quantitative RT-PCR**

Kidney tissue was homogenized and total RNA was isolated using a Trizol reagent (Invitrogen) and followed by Ambion DNA-free deoxyribonuclease treatment (Life Technologies). First-strand cDNA synthesis from 250 ng of total RNA was
performed using the SuperScript III first-strand kit (Invitrogen, Life Technologies). Quantitative PCR reactions were carried out with the primer sets listed in Supplemental Table 1. Quantitative PCR amplification was performed on Applied Biosystems 7900HT Fast real-time PCR system using Sybr Green reaction mix and was analyzed using SDS Automation Controller software (version 2.3; Applied Biosystems, Life Technologies) and normalized to levels of a housekeeping mRNA (18S rRNA and glyceraldehyde-3-phosphate dehydrogenase as appropriate).

Relative quantification of gene expression was calculated using the formula $2^{-\Delta\Delta Ct}$ (43). Each individual sample was twice reverse transcribed and assessed by quantitative PCR. The average of the two values obtained for each sample was used to determine whether the mean of gene expression was significantly different between genotypes. For each group ($n = 8$), data are presented as mean ± SEM.

Analysis of body composition by dual-energy x-ray absorptiometry (DEXA)

Body composition was assessed by a DEXA scan (PIXImus2; Lunar/GE Medical Systems). Mice were anesthetized by exposure to 2%–3% isoflurane-oxygen gas and placed on the scanner bed in the prone position. The PIXImus was calibrated using an aluminum/lucite phantom (corresponding to bone mineral density of 0.0592 g/cm² and 12.5% fat) on each day of testing according to the manufacturer’s instructions. One scan per mouse was performed and analyzed with PIXImus software (2.10; GE/Lunar). The head was excluded from calculation using a manual region of interest (ROI). To evaluate abdominal fat, a ROI was defined from the whole-body scan: from the apical border of the pelvis to the caudal border of the ribs as was validated previously (44).

Statistics

All data sets were analyzed by a two-way ANOVA and Tukey’s multiple comparison posttests to identify significant effects between groups (GraphPad Prism, version 6.0a; GraphPad Software). The mean difference was considered significant at $P < .05$. All data are reported as mean ± SEM.

Results

Mutagenesis

The C603S mutation in the mouse MR gene, which is equivalent to the C604S DBD mutation in the rat MR characterized by Pearce et al (40), was successfully introduced into the first zinc finger of the DBD to eliminate DNA binding. The properties of the MR C603S mutation were confirmed in vitro (Figure 2) in which both protein levels and ligand binding were intact relative to the wild-type MR, but transactivation was abolished, as also described by Pearce et al (40).

The domain structure of the MR and the sequence of the DBD with the mutation are shown in Figure 1A. Mice heterozygous for the mutation were successfully generated (Figure 1, B and C). Male and female mice heterozygous for the C603S mutation were crossed to yield homozygous C603S mice. Although these were born at the normal frequency (~25%), they lost weight and died between days 10 and 13 (Figure 3). To exclude the possibility that the neomycin resistance gene cassette inserted downstream of exon 3 impaired expression of the MR, the heterozygous mice were crossed with Flp-deleter mice to remove the
cassette and then bred to heterozygosity. These mice were then crossed to again generate mice homozygous for the MR C603S mutation but now devoid of the neomycin cassette. They exhibited essentially the same phenotype as the strain with the neomycin cassette. All subsequent studies were performed with the line lacking the neomycin resistance gene cassette.

MR C603S expression

The phenotype observed is similar to that reported for the MR null mice by Berger et al (2). To demonstrate that we have not simply generated an MR null allele and recapitulated the MR null mouse, it was important to establish not only that the MR C603S mutation was present in the germline of the mice but also that there are no additional mutations in the MR and that the MR mRNA both contains the mutation and the levels are not subject to missense mediated decay, ie, MR protein levels are preserved. Kidney mRNA was reverse transcribed and the DNA sequenced from four wild-type and three MR C603S mutant mice to confirm the presence of the C603S mutation (Figure 4A). Quantitative RT-PCR indicated that MR levels were equivalent in the kidney of the wild-type and mutant mice (Figure 4B). To exclude additional mutations, the full coding region of the MR cDNA from a wild-type mouse and a C603S mutant mouse was sequenced. Both were aligned with the published mouse MR cDNA referenced sequence (NM_001083906.1) with which they were identical except for the thymine to adenosine charge at codon 603 in the MR C603S mRNA.

Immunohistochemical staining for the MR in the kidney suggested, at least on a semiquantitative basis, that MR protein levels were equivalent for the wild-type and mutant mice. The pattern of staining in the distal convoluted tubules (Figure 4C) of the wild-type mice, as described previously (6), was also observed in the mutant mouse kidney. Protein levels of MR C603S, determined in kidney extracts by Western blot analysis, were also equivalent to the wild-type MR levels (data not shown). Both the levels and localization observed are consistent with the findings of Pearce et al (40) in vitro in which although nuclear localization was diminished for the MR with the C603S mutation, protein levels were essentially equivalent. In the MR C603S mice, the levels of a number of MR-regulated genes, including the epithelial sodium channel (ENaC) subunit genes, were examined; however, they did not differ between wild-type and mutant mice, consistent with responses in the MR null mouse (2).

Saline rescue of the C603S mice

The goal of the C603S mutant model was to examine the role of nonclassical MR signaling in a range of tissues, which is clearly thwarted by the observed postnatal lethal phenotype. To overcome this limitation, we used the approach of Bleich et al (45), with the MR null mice and administered sc isotonic saline to the entire litter at day 5 with twice-daily injections through to the time of weaning. This strategy enabled survival of a majority of the mice (Figure 5A), albeit they remained consistently smaller (Figure 5B). This likely in part reflects dehydration, although we were not able to determine the total body water content of the mice. They also appeared to have, on macroscopic inspection, a loss of abdominal fat deposits, leading us to formally quantify this using DEXA. These data show that, although the mutant mice do indeed have markedly diminished fat mass, it is proportionate to the levels of lean mass and the body weight (Figure 6). Analyses based on the abdominal area (ROI) showed only equivalent results (data not shown).

Corticosteroid levels

The plasma levels of both aldosterone and corticosterone were measured in 28- to 35-day-old mice (Figure 7). The levels of aldosterone, as might be expected, are elevated in the heterozygote mice relative to wild-type mice and in the mice homozygous for the C603S mutations relative to both. The levels appear relatively high, but this may reflect the use of a RIA, which may result in higher values than an liquid chromatography-mass spectrometry-based method; the key issue here is the relative levels wild-type MR vs MR C603S. Plasma corticosterone levels, although elevated, presumably reflecting stress in the mice, were not significantly different between groups.
Gene expression

The expression of the MR, GR, and a range of known MR-regulated genes (1, 46) was examined in wild-type and rescued MR C603S mutant mice of both sexes (Figure 8). No significant difference in MR or GR mRNA levels was observed. Renin levels were markedly elevated in the rescued young adult mutant mice. The ENaC subunit mRNA levels were not different, nor unsurprisingly were the serum and glucocorticoid regulated kinase (sgk1), glucocorticoid-induced leucine zipper (gilz), and connector enhancer of kinase suppressor of Ras isoform 3 (cnkrs3) genes. By contrast, the corticosteroid-induced factor (CHIF) gene was significantly elevated in the MR C603S mutant mice. Other putatively MR regulated genes, FKBP5, period 1, and period 2 (data not shown), were not significantly different between wild-type and mutant mice.

Discussion

The relative importance of the role of direct DNA binding in MR-mediated signaling has not previously been explored, in contrast to other steroid hormone receptors. To address this question, we inserted a well-characterized DNA-binding mutation into a transgenic mouse line, which was bred to homozygosity. The clear implication of these data is that the DNA binding function of the receptor is absolutely required for regulation of epithelial sodium transport. The impact of the MR C603S mutation, with retention of expression and ligand binding but with loss of transactivation, was confirmed in vitro. To ensure that in the construction of these mice other mutations had not been introduced into the MR and that in vivo this mutation had not induced nonsense mediated decay, the status of the mouse model was characterized in detail. The presence of the homozygous MR C603S mutation was confirmed in both the germline DNA of these mice and in the MR mRNA expressed in the kidney. The entire coding region of the MR mRNA was sequenced to con-
firm its integrity, and mRNA levels were found to be equivalent in the kidney of both wild-type and mutant mice. The same is also true of the heart (data not shown).

The presence and spatial distribution of the MR protein was determined by immunohistochemistry in mice treated with 1% saline for 8 days during the critical period of salt wasting. Semiquantitative analysis of MR expression was very similar between genotype, whereas the subcellular distribution of the receptor showed less nuclear localization, consistent with the in vitro data for this mutation (40). As might be predicted from the phenotype, renin mRNA levels were markedly elevated at this time. Although ideally one would seek to confirm in vitro and/or in vivo that both transrepression and nongenomic signaling are intact, this is challenging; indeed, the intent of this model is to provide a discovery tool for these end points.

The crystal structure of the MR DBD coupled with DNA has only recently been determined for the MR (12). It has only very subtle differences from the GR DBD structure (47). Cysteine 603 is the first of the four cysteine residues that coordinate with a zinc atom to form the first of the two so-called zinc fingers. Loss of this structural motif would be anticipated to be devastating for the folding of the finger. The functional consequences observed are certainly consistent with this conclusion.

Although a significant impact for this mutation on renal MR action was not unanticipated, it was hoped that the mice might be used to explore the nature of MR signaling in other tissues in which it plays a key physiological role unrelated to sodium homeostasis or indeed aldosterone. We therefore sought to rescue these mice using the approach of Bleich et al (45) with saline administered to the pups twice daily from day 5. This approach was partially successful, although the number of mice obtained was limited. Rescued mutant mice remain smaller than the littermate controls (who were also treated with saline), and although their adipose deposits appear depleted, an observation of some interest, given the role of the MR in adipocyte differentiation (8), formal analysis of body composition using DEXA revealed the loss to be proportionate to other parameters of body composition.

Aldosterone levels were, as might be anticipated, markedly elevated, noting that the levels in the wild-type group who also received saline were likely to be lower than normal. The renal renin mRNA levels are also elevated, consistent with activation of the renin-angiotensin-aldosterone systems in the face of ongoing salt loss. Although the dehydration observed is likely to reflect sodium depletion with consequent hypovolemia and hypotension (45), an effect of MR action in the vascular may also be an important contributor (48, 49).

We also examined the expression of a range of genes known or thought to be directly regulated by MR activation. We had anticipated that their mRNA levels may be low relative to control in which regulation was through the classical DNA-binding mechanisms, whereas genes regulated by the MR independent of the DBD might be expected to be elevated. As with the MR null mice (2), the expression of the principal epithelial channel subunit gene, ENaC, did not differ between genotype at 8 days or in the rescued mice. In contrast, Zhang et al (50) recently reported that renal ENaC mRNA levels were in fact lower in the MR null mice at day 5; this time point was not examined in the MR C603S mice. Similarly, key aldosterone-induced genes involved in the regulation of the turnover of ENaC, sgk1, CNKSR3, and gilz did not differ by genotype despite the elevated aldosterone levels and is consistent with the failure of MR signaling induced by the C603S mutation. An important caveat, as noted above, is that the wild-type mice are also on saline, which may explain why the anticipated relative decrement in the expression of these genes in the mutant mice is not observed.
By contrast, the CHIF gene, which has previously been reported to be aldosterone regulated (51, 52), was expressed at higher levels in the C603S mice. CHIF is a member of the FXYD family of single-transmembrane domain proteins, which act to modulate cell membrane ion transporters (53). CHIF is thought to increase the affinity of NaK-ATPase for sodium and hence increase sodium efflux at the basolateral membrane, a key component of vectorial sodium transport (54). Although CHIF, as with many of these genes, can also be regulated by activation of GR signaling (52), neither GR mRNA levels nor corticosterone levels are elevated in the C603S mice relative to the wild-type mice. In contrast to the other aldosterone-induced genes examined, hormone response elements in the CHIF gene have not been identified. The acute response of CHIF to aldosterone is, however, regulated at the level of transcription (55), and CHIF knockout mice exhibit a mild decrease in sodium transport in response to glucocorticoid treatment or sodium depletion (56). These findings suggest that in the C603S mice, either CHIF is regulated independently of a hormone response element and DNA-binding through either an interaction with another transcription factor or via a membrane associated mechanism or else the up-regulation observed is secondary to the consequence of the loss of MR signaling, particularly the sodium wasting. Two caveats that should be noted are that CHIF is predominantly regulated by corticosteroids and sodium depletion in the distal colon rather than in the kidney and that it is most abundantly expressed in the renal papilla/medulla in which its regulation might be anticipated to be independent of aldosterone (57).

In considering the significance of these changes in gene expression, the differential response of a gene that might be expected to be coordinately regulated is a key finding. An important control would be to contrast the change in CHIF gene expression with the MR null mice; however, these mice were not available at the time of the study and the logistics of resourcing and maintaining two lines with profound salt wasting is daunting. The original molecular characterization of the MR null mice involved only analyses of ENaC subunit genes (2) as discussed above. The use of similar models for other steroid receptors has been both revealing and controversial. The first of these was a study in which a mutation was introduced into the DBD of the GR at a position that would preclude the formation of the GR homodimers, the GR dim mouse (36). They reported loss of the classical metabolic responses to dexamethasone with retention of the antiinflammatory response, arguing that glucocorticoid response element-mediated signaling had been abrogated with transrepression retained. Subsequent studies, however, have challenged these conclusions, and the situation with the GR
dim mouse is clearly more complex than originally thought (58, 59).

Jakacka et al (37) subsequently created analogous dimerization-deficient mice for the ER. They mutated two amino acids in the second knuckle of the first zinc finger, the P-box of the mouse ERα DBD, a region involved in interactions with the estrogen response element. The resulting phenotype suggested unexpected consequences of nonclassical ER signaling. A second model involving ERα introduced four mutations into this region, resulting in a phenotype rather more closely resembling that of the ERα null mouse (60). Recently Hewitt et al (61) have resolved the dichotomy between these two models by demonstrating that the DNA-binding mutation described by Jakacka et al (37) actually changes the specificity of the DNA binding such that it can activate progesterone response elements. Therefore, as with the GR dim mouse, caution is required when interpreting such models. The MR C603S mouse we describe is consistent with the second ERα DBD-mutant mouse (60) in which DNA binding would appear to be truly abrogated.

Although we believe the C603S MR mouse to be a valid model of loss of MR DNA binding with the potential to elucidate the nuances of nonclassical MR signaling, the profound salt wasting makes this challenging. In preliminary studies we have sought to harness the power of the model to explore the role of nonclassical signaling in nonrenal tissues by crossing these mice with tissue-specific MR null mice (6, 7, 42) to identify tissue-specific nonclassical signaling. These early studies suggest the retention of specific MR signaling, which therefore represents a putative DNA-binding-independent pathway (Young, Morag J., unpublished observation).

In conclusion, we have established the central role of classical MR signaling in aldosterone-induced, MR-mediated epithelial sodium transport. We have also identified an MR-induced gene whose regulation may not be entirely mediated by classical MR signaling. Finally, although not without its challenges, this model has the potential to identify the relative contribution of nonclassical MR signaling in specific nonepithelial tissues.

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