Transcriptional activation of elephant shark mineralocorticoid receptor by corticosteroids, progesterone, and spironolactone

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The mineralocorticoid receptor (MR) is a nuclear receptor and part of a large and diverse family of transcription factors that also includes receptors for glucocorticoids, progesterone, androgens, and estrogens. The corticosteroid aldosterone is the physiological activator of the MR in humans and other terrestrial vertebrates; however, its activator is not known in cartilaginous fish, the oldest group of extant jawed vertebrates. Here, we analyzed the ability of corticosteroids and progesterone to activate the full-length MR from the elephant shark (Callorhinichus milii). On the basis of their measured activities, aldosterone, cortisol, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol, progesterone, and 19-norprogesterone are potential physiological mineralocorticoids. However, aldosterone, the physiological mineralocorticoid in humans and other terrestrial vertebrates, is not found in cartilaginous or ray-finned fish. Although progesterone activates MRs in ray-finned fish, progesterone does not activate MRs in humans, amphibians, or alligator, suggesting that during the transition to terrestrial vertebrates, progesterone lost the ability to activate the MR. Both elephant shark MR and human MR are expressed in the brain, heart, ovary, testis, and other nonepithelial tissues, suggesting that MR expression in diverse tissues evolved in the common ancestor of jawed vertebrates. Our data suggest that 19-norprogesterone– and progesterone-activated MR may have unappreciated functions in reproductive physiology.

INTRODUCTION

The mineralocorticoid receptor (MR) belongs to the nuclear receptor family, a large and diverse group of transcription factors that also includes receptors for glucocorticoids, progesterone, androgens, and estrogens. The MR and other steroid receptors have a characteristic modular structure consisting of an N-terminal domain (NTD) (domains A and B), a central DNA binding domain (DBD; domain C), a hinge domain (domain D), and a C-terminal ligand-binding domain (LBD) (domain E) (Fig. 1) (2, 4, 23–25). The LBD alone is competent to bind steroids. Aldosterone (Aldo) is the physiological activator of transcriptional activity of human MR in epithelial tissues, such as the kidney distal collecting tubules and the colon, in which the MR regulates electrolyte homeostasis, its classical function. The physiological function of the MR in these tissues is still being elucidated (14, 18, 20, 22).

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Fig. 1. Comparison of the domains in elephant shark MR with those in vertebrate MRs. MRs from elephant shark (shark), zebrafish, coelacanth, Xenopus, chicken, and human were compared. The functional A/B domain through to the E domain is schematically represented with the numbers of amino acid residues. The percentage of amino acid identity is depicted. GenBank accession numbers are as follows: elephant shark MR (XP_007902220), zebrafish MR (NP_001093873), coelacanth MR (XP_014348128), Xenopus MR (NP_001084074), chicken MR (AC037437), and human MR (NP_000892).
Fig. 2. Structures of steroids that are ligands for the MR. Aldo, 11-deoxycorticosterone, and 11-deoxycortisol are physiological mineralocorticoids in terrestrial vertebrates (4, 6, 12, 76). 11-deoxycortisol is both a mineralocorticoid and a glucocorticoid in lamprey (4, 77), whereas 19norProg is a weak agonist for rat MR (85, 86). 19norProg and Spiron are agonists for fish MRs (25, 37, 45), whereas 19norProg is a weak agonist for rat MR (54, 55). All of the ligands shown here have a ketone at C3 and, thus, are called 3-ketosteroids.
corticosterone, 11-deoxycortisol, cortisol, Prog, 19norProg, 17-hydroxyprogesterone (17OH-Prog), and Spiron. We found that all 3-ketosteroids, including Prog, had a half-maximal response (EC$_{50}$) of 1 nM or less for full-length elephant shark MR. Activation by Prog, 19norProg, and Spiron of truncated elephant shark MR resembled that of zebrafish MR, but not chicken MR, indicating that the activation of MR by Prog is an ancestral response, conserved in cartilaginous fish and ray-finned fish, but lost in Xenopus, alligator, and human MRs, and distinct from the activation of chicken MR, which arose independently. We investigated the relative expression of elephant shark MR by using RNA-seq data and found widespread expression of MR in various elephant shark tissues (gill, kidney, heart, intestine, liver, spleen, brain, ovary, and testis). This suggests that the widespread expression of human MR in tissues, such as the brain, heart, liver, spleen, ovary, and testis, in which the MR does not regulate electrolyte homeostasis, evolved early in vertebrate evolution, in a common ancestor of jawed vertebrates. The abundant MR expression in ovary and testis suggests a role for 19norProg-MR and Prog-MR complexes in elephant shark reproduction, as well as other unappreciated functions in some other MR-containing tissues. Last, our data suggest that several 3-ketosteroids, including Prog, may have been ancestral mineralocorticoids.

**RESULTS**

**Functional domains of elephant shark MR and other vertebrate MRs**

We first compared the functional domains of elephant shark MR to those of selected vertebrate MRs (Fig. 1). Elephant shark MR and human MR have 92 and 67% identity in their DBDs and LBDs, respectively. Furthermore, elephant shark MR has similar conservation to the DBDs (91 to 92%) and LBDs (64 to 69%) of other MRs. The A, B, and D domains of elephant shark MR and those of other MRs are much less conserved (Fig. 1).

**Transcriptional activation of full-length and truncated elephant shark MR by corticosteroids, Prog, and Spiron**

We screened a panel of steroids at two different concentrations (0.1 and 1 nM) for their ability to stimulate the transcriptional activity of full-length and truncated elephant shark MR. At 1 nM, Aldo, cortisol, corticosterone, 11-deoxycorticosterone, and 11-deoxycortisol activated full-length elephant shark MR (Fig. 3A), indicating that elephant shark MR has broad specificity for corticosteroids. Furthermore, at 1 nM, 19norProg had activity comparable to that of the five corticosteroids, whereas Prog and Spiron had intermediate activity and 17OH-Prog had little activity (Fig. 3A). In parallel experiments, truncated elephant shark MR, lacking the A/B domain and containing a GAL4-DBD instead of the MR DBD, retained responsiveness to all corticosteroids and to 19norProg (Fig. 3B). However, Prog and Spiron had reduced activity, and 17OH-Prog had little activity for truncated elephant shark MR.

**EC$_{50}$ values for steroid activation of elephant shark MR**

We next determined the concentration dependence of transcriptional activation of full-length elephant shark MR by corticosteroids (Aldo, cortisol, corticosterone, 11-deoxycorticosterone, and 11-deoxycortisol; Fig. 4A) and by Prog, 19norProg, 17OH-Prog, and Spiron (Fig. 4C). We also determined the corresponding concentration-dependent curves for activation of truncated elephant shark MR (Fig. 4, B and D). We then calculated the EC$_{50}$ values of corticosteroids for full-length and truncated elephant shark MR (Table 1). The five corticosteroids, Aldo, corticosterone, 11-deoxycorticosterone, cortisol, and 11-deoxycortisol, had similar EC$_{50}$ values and fold activation of full-length elephant shark MR (Fig. 4A). The EC$_{50}$ values varied from 0.063 nM for 11-deoxycorticosterone to 0.46 nM for cortisol (Table 1). EC$_{50}$ values of <1 nM are consistent with each corticosteroid being a physiological activator of elephant shark MR. Fold activation compared to that of Aldo (which was set at 100%) varied from 83% for 11-deoxycorticosterone and 11-deoxycortisol to 114% for cortisol (Fig. 4A and Table 1), indicating that all five corticosteroids are activators of elephant shark MR.

Truncated elephant shark MR was also substantially activated by corticosteroids, which had EC$_{50}$ values ranging from 0.024 nM for 11-deoxycorticosterone to 0.19 nM for cortisol (Table 1). Fold activation compared to that of Aldo (100%) decreased to 79% for cortisol, 81% for 11-deoxycorticosterone, and 77% for 11-deoxycortisol.
We also compared these EC₅₀ values to the previously determined EC₅₀ values of corticosteroids for full-length and truncated human, chicken, alligator, *Xenopus*, and zebrafish MRs (36) and for skate MR (49). Unlike elephant shark MR, there are differences among terrestrial vertebrate MRs in their EC₅₀ values and in their fold activation for the five glucocorticoids. Whereas Aldo and corticosterone had similar nanomolar or lower EC₅₀ values for the transcriptional activation of full-length and truncated terrestrial vertebrate MRs, 11-deoxycorticosterone and 11-deoxycortisol had higher EC₅₀ values for the activation of truncated human, alligator, and *Xenopus* MRs, compared to their EC₅₀ values for the activation of the corresponding full-length MRs (Table 1) (36). Furthermore, all corticosteroids had low EC₅₀ values and resulted in substantial fold activation of full-length and truncated zebrafish MR (Table 1) (36), suggesting that zebrafish MR retains responses to corticosteroids found in elephant shark MR.

Among the progestins, we found that 19norProg was the most active for full-length and truncated elephant shark MR (Fig. 4D and Table 2). 19norProg had EC₅₀ values of 0.43 and 0.018 nM for full- and truncated elephant shark MR, respectively. These values are comparable to the EC₅₀ values of corticosteroids for full-length and truncated elephant shark MR. The fold activation by 19norProg of full-length and truncated elephant shark MR was 84 and 98%, respectively, compared to that of Aldo, suggesting that 19norProg could be a physiological activator of elephant shark MR. Prog and Spiron had EC₅₀ values of 0.27 and 0.66 nM, respectively, for full-length elephant shark MR. However, their fold activation of full-length MR was about 45% of that of Aldo. 17OH-Prog had an EC₅₀ value of 1.9 nM for full-length MR and exhibited only 25% of the fold activation induced by Aldo.

Previously, we reported that Prog, 19norProg, and Spiron are transcriptional activators of full-length and truncated chicken and zebrafish MRs (36). However, the EC₅₀ values of Prog, 19norProg, and Spiron for these MRs were not determined. We have remedied this omission and now report their EC₅₀ values, as well as the EC₅₀ values for 17OH-Prog (Fig. 5 and Table 2), for full-length and truncated elephant shark MR for comparison. With respect to full-length chicken MR, Prog and 19norProg had EC₅₀ values of 0.68 and 0.71 nM, respectively, which would be expected to be sufficient for the physiological activation of chicken MR (Fig. 5A and Table 2). The fold activation of full-length chicken MR by Prog and 19norProg was 62 and 68%, respectively, compared to that of Aldo. 17OH-Prog had an EC₅₀ value of 29 nM, and its fold activation of the receptor was 15% of that of Aldo. The EC₅₀ value of Spiron for full-length chicken MR was 5.1 nM. The extent of activation of truncated chicken MR by Prog, 19norProg, 17OH-Prog, and Spiron was too low for the calculation of EC₅₀ values (Fig. 5C and Table 2), which suggests that allosteric interactions between the NTD and LBD are important in the activation of chicken MR by progestins.

With respect to zebrafish MR, 19norProg had an EC₅₀ value of 0.9 nM and exhibited fold activation of the receptor that was 83% of that of Aldo, whereas Prog had an EC₅₀ value of 2.4 nM and gave 77% of the fold activation induced by Aldo for full-length zebrafish MR. These responses are sufficient for both 19norProg and Prog to be physiological activators of zebrafish MR. In contrast, 17OH-Prog had an EC₅₀ value of 18 nM and its fold activation of the full-length zebrafish MR was only 44% of that of Aldo. The EC₅₀ values of all progestins and Spiron for truncated zebrafish MR were greater than their EC₅₀ values for full-length zebrafish MR. The EC₅₀ values of Prog and 19norProg for the truncated zebrafish MR were 98 and 64 nM, respectively, which suggests that they are less effective at activating the truncated receptor than they are at activating full-length
zebrafish MR. The extent of activation of truncated zebrafish MR by 17OH-Prog and Spiron was too small for their EC$_{50}$ values to be calculated. This contrasts to the more substantial activation by corticosteroids of full-length zebrafish MR (Fig. 5 and Tables 1 and 2), suggesting that allosteric interactions between the NTD/DBD and LBD contribute to the activation of the zebrafish MR by progestins.

RNA-seq analysis of elephant shark MR
We examined the relative expression of elephant shark MR (NR3C2) mRNA in 10 tissues based on previously published RNA-seq data (Fig. 6A) (9). The NR3C2 gene was expressed widely in all tissues, including the gills and kidney, two traditional mineralocorticoid-responsive tissues. Furthermore, there was considerably higher expression in the ovary and testis, the two reproductive tissues analyzed.

RNA-seq analysis of human MR
Analysis of previously published RNA-seq data for the human MR (Fig. 6B) (50) revealed that the NR3C2 is expressed in the kidney, colon, brain, heart, liver, ovary, spleen, and testis. This pattern of expression of human MR in diverse tissues is similar to that of elephant shark NR3C2.

**DISCUSSION**
Cartilaginous fish, including elephant sharks, occupy a key position in the evolution of vertebrates as an out-group to ray-finned fish, the largest group of extant vertebrates, and the lobe-finned fish, which are the forerunners of terrestrial vertebrates. Furthermore, the elephant shark genome is evolving slowly (9), making it attractive for studying ancestral proteins, including the MR, which first appeared as a distinct MR ortholog in cartilaginous fish (5, 6, 10, 49).

Our investigation of corticosteroid activation of elephant shark MR revealed that Aldo, corticosterone, 11-deoxycorticosterone, cortisol, and 11-deoxycortisol had EC$_{50}$ values of <1 nM for full-length elephant shark MR (Fig. 4 and Table 1). Prog, 19norProg, and Spiron also had subnanomolar EC$_{50}$ values for full-length elephant shark MR. In addition to their low EC$_{50}$ values, all of these corticosteroids and 19norProg exhibited substantial fold activation of the transcriptional activity of full-length MR (Fig. 4, A and C), whereas Prog was about 43% as effective as Aldo. Several corticosteroids, as well as 19norProg and Prog, are potential physiological mineralocorticoids for elephant shark MR. Compared to their EC$_{50}$ values for full-length elephant shark MR, the EC$_{50}$ values of all five corticosteroids and 19norProg for the truncated MR were reduced, whereas...
the EC$_{50}$ value for Spiron was slightly less, and the EC$_{50}$ values for Prog and 17OH-Prog were about twofold greater (Tables 1 and 2). Regarding the truncated skate MR, most of the EC$_{50}$ values of the corticosteroids (49) are similar to that for elephant shark MR (Table 1). The exception is 11-deoxycortisol, whose EC$_{50}$ value was greater than 200-fold higher for skate MR than for elephant shark MR.

Prog, 19norProg, or both may be mineralocorticoids in cartilaginous fish

The Prog concentration in female elephant shark serum is 4.4 ng/ml (14 nM) (51). In draughtboard sharks (*Cephaloscyllium laticeps*), the serum concentration of Prog in females is 8 ng/ml (25.4 nM), whereas in males it is 1 ng/ml (3.2 nM) (52). In female zebrafish (*Stegostoma fasciatum*), the serum concentration of Prog is 10 ng/ml (31.8 nM) (53). Together, these data suggest that Prog concentrations are sufficient to activate the MR in cartilaginous fish. We found that 19norProg has an EC$_{50}$ value of 0.043 nM for elephant shark MR. Moreover, 19norProg evoked a stronger response from elephant shark MR than did Aldo (Fig. 4, A and B). C19 demethylase, which removes the C19 methyl group from steroids, has been detected in the mammalian kidney (54). If C19 demethylase is present in elephant shark, then 19norProg should be considered as a potential physiological mineralocorticoid.

### Table 2. EC$_{50}$ values for the activation by Prog and Spiron of full-length and truncated (LBD) constructs of elephant shark, zebrafish, and chicken MRs.

| MR              | Aldo     | Prog     | 17OH-Prog | 19norProg | Spiron |
|-----------------|----------|----------|-----------|-----------|--------|
| **Elephant shark full** | 1.1 × 10$^{-10}$ | 2.7 × 10$^{-10}$ | 1.4 × 10$^{-9}$ | 4.3 × 10$^{-11}$ | 5.5 × 10$^{-10}$ |
|                 | 100%     | 43%      | 25%       | 84%       | 45%    |
| **Elephant shark LBD** | 3.7 × 10$^{-11}$ | 4.8 × 10$^{-10}$ | 2.9 × 10$^{-9}$ | 1.8 × 10$^{-11}$ | 4.2 × 10$^{-10}$ |
|                 | 100%     | 40%      | 26%       | 98%       | 53%    |
| **Zebrafish full** | 8.2 × 10$^{-11}$ | 2.4 × 10$^{-9}$ | 1.8 × 10$^{-8}$ | 9.4 × 10$^{-10}$ | 3.8 × 10$^{-9}$ |
|                 | 100%     | 77%      | 44%       | 83%       | 54%    |
| **Zebrafish LBD** | 2.7 × 10$^{-11}$ | 9.8 × 10$^{-8}$ | *         | 6.4 × 10$^{-8}$ | *      |
|                 | 100%     | 122%     | 24%$^1$   | 122%      | 73%$^1$ |
| **Chicken full** | 6.2 × 10$^{-11}$ | 7.1 × 10$^{-10}$ | 2.9 × 10$^{-8}$ | 6.8 × 10$^{-10}$ | 5.1 × 10$^{-9}$ |
|                 | 100%     | 62%      | 15%       | 68%       | 30%    |
| **Chicken LBD** | 1.3 × 10$^{-10}$ | *        | *         | *         | *      |
|                 | 100%     | 21%$^1$  | —         | 29%$^1$   | —      |

$^1$ Curve did not saturate.  
$^1$ Relative induction at 1 μM compared to Aldo.

![Fig. 5. Concentration-dependent transcripational activation of full-length and truncated chicken and zebrafish MR by Prog, 19norProg, and Spiron.](https://stke.sciencemag.org/)
Although in cell-based assays, 19norProg is an agonist for elephant shark, zebrafish, and chicken MRs (Table 2), and 19norProg at 1 nM is an antagonist for human MR (36, 48). This finding contrasts to that from in vivo studies in rats, which found that 19norProg is an MR agonist with about 100-fold weaker activity than that of Aldo (54, 55). A possible explanation for this difference is that in rats, 19norProg is metabolized to 11β-deoxycorticosterone, which is an MR agonist (56–58). Another possibility, based on the ability of 11β-hydroxyprogesterone to activate human MR (28), is that 19norProg is metabolized to 11β-hydroxy19norProg.

We propose that the transcriptional activation of elephant shark MR by 19norProg, as well as by Prog and Spiron, can be explained by the discovery by Geller et al. (48) that the S810L mutant MR is activated by 1 nM Prog, 19norProg, and Spiron, unlike wild-type human MR, for which these steroids are antagonists. On the basis of a three-dimensional model of the S810L mutant MR, Geller et al. (48) proposed that a contact between Leu810 and Ala773 was sufficient for transcriptional activation of the S810L mutant MR by Prog and 19norProg. This motivated Geller et al. (48) to construct the S810M mutant human MR, which was activated by 19norProg. Elephant shark MR and skate MR contain a methionine at the position corresponding to Ser810 and an alanine corresponding to Ala773 (Fig. 7) (25). On the basis of the model of Geller et al., we propose that the transcriptional activation of elephant shark MR by 19norProg is due to a contact between Met782 (helix 5) and Ala745 (helix 3), which would stabilize the A ring of 19norProg, promoting transcriptional activation of the receptor.

A potential role for elephant shark MR in reproductive physiology

The EC50 value of Prog for elephant shark MR (0.27 nM), the physiological concentration of Prog of 14.5 nM (51), and the abundant expression of the MR in the elephant shark ovary and testis (Fig. 6A) all suggest that a Prog-MR complex may be important in reproductive responses in elephant shark. Of course, Prog also acts as a reproductive steroid in the ovary and testis through its transcriptional activation of the PR (39, 60). On the basis of evidence that Prog activates the MR in several ray-finned fish (25, 37, 45, 47), a Prog-MR complex may also be active in reproductive tissues and other tissues in ray-finned fish, as well as in cartilaginous fish.

RNA-seq analysis found MR expression in elephant shark gills and kidneys (Fig. 6A), two classical targets for the MR-mediated regulation of electrolyte transport (6). Moreover, RNA-seq analysis also identified MR expression in elephant shark heart and brain, two other tissues in which corticosteroids exert important physiological actions through the mammalian MR (16–18, 20–22, 61–64). MR antagonists are useful in treating heart failure (21, 65), although their mechanism of action is not fully understood (21, 64). A study by Oakley et al. (64) provides insights into how inhibition of MR activity in the heart affects the survival of damaged cardiomyocytes in mice. Oakley et al. (64) reported that the balance between
the actions of the MR and its close relative, the GR, influences whether damaged cardiomyocytes die or survive. 

RNA-seq analysis of elephant shark MR indicates that the expression of the MR in diverse tissues was conserved during the descent from cartilaginous fish to humans. Expression of shark MR in many tissues (brain, heart, liver, and ovary) in which the MR is not likely to regulate electrolyte homeostasis, the classical function of the MR, further supports evidence from the last 30 years (3, 17–19, 21, 61, 63, 65–67) that mineralocorticoid activity is an incomplete functional description of this nuclear receptor. An alternative name is needed to describe more completely the functions of the MR.

MATERIALS AND METHODS
Chemical reagents
Aldo, cortisol, corticosterone, 11-deoxy corticosterone, 11-deoxycortisol, Prog, 19norProg, 17OH-Prog, and Spiron were purchased from Sigma-Aldrich. For reporter gene assays, all hormones were dissolved in DMSO; the final DMSO concentration in the culture medium did not exceed 0.1%.

Construction of plasmid vectors
The full-coding regions from elephant shark MR were amplified by polymerase chain reaction (PCR) with KOD DNA polymerase. The PCR products were gel-purified and ligated into pcDNA3.1 vector (Invitrogen) for the full-coding region or into the pBIND vector for D and E domains (68).

Elephant shark MR gene expression analysis
We had previously generated RNA-seq for several tissues of elephant shark as part of the elephant shark genome project (9) and submitted them to National Center for Biotechnology Information (accession number SRA054255). We downloaded RNA-seq reads for the brain, gills, heart, intestine, kidney, liver, muscle, ovary, spleen, and testis, and assembled each of them into transcripts using the program Trinity version r2013-08-14 (69). The assembled transcripts were used to determine the extent of expression of the NR3C2 gene. To determine the relative expression of the MR gene, we performed abundance estimation of transcripts from the aforementioned 10 tissues. Trinity transcripts from all 10 tissues and the full-length complementary DNA sequence of the MR gene were combined together and clustered using CD-HIT v4.6.1 at 100% identity (70). RNA-seq reads from each of the 10 tissues were independently aligned to the clustered transcript sequences, and the abundance of MR transcripts was estimated by RSEM v1.2.25 (71), which uses bowtie v2.2.6 for aligning (72). Transcript abundances were measured in terms of normalized counts called FPKM (71). FPKM is estimated by normalizing the gene length, followed by normalizing for sequencing depth.

Transactivation assay and statistical analysis
Transfection and reporter assays were performed with HEK 293 cells, as described previously (68, 73). All experiments were performed in triplicate. The values shown in the figures are means ± SEM from three separate experiments, and the dose-response data and EC50 values were analyzed and calculated with GraphPad Prism. Comparisons between two groups were performed using the Student’s t test, and all multigroup comparisons were performed by one-way analysis of variance (ANOVA), followed by Bonferroni test. P < 0.05 was considered to be statistically significant. The use of HEK 293 cells and an assay temperature of 37°C does not replicate the physiological environment of elephant sharks. Nevertheless, studies with HEK 293 cells and other mammalian cell lines have proven useful for other studies of transcriptional activation by corticosteroids of skate MR (49) and teleost fish (37, 45, 74, 75) MRs.

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Transcriptional activation of elephant shark mineralocorticoid receptor by corticosteroids, progesterone, and spironolactone
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Expanding mineralocorticoid functions
Mineralocorticoid receptors (MRs) belong to the nuclear receptor family of transcription factors. Aldosterone is a physiological ligand for the human MR, which is best known for regulating electrolyte homeostasis. Noting that the MR first arose in cartilaginous fish, which do not have aldosterone, Katsu et al. examined the binding and activity profiles of a range of corticosteroids and steroid hormones for the MR of the elephant shark, a cartilaginous fish found in the oldest group of jawed vertebrates. These studies suggest that elephant shark MR is activated by progesterone, which acts as an antagonist of the human MR. Given the abundance of the MR in elephant shark ovaries and testis, these findings suggest that the MR may play an unappreciated role in reproductive physiology.