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The Severe Form of Hypertension Caused by the Activating S810L Mutation in the Mineralocorticoid Receptor Is Cortisone Related

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A gain of function mutation resulting in the substitution of leucine for serine at codon 810 (S810L) in the human mineralocorticoid receptor (MR) is responsible for early-onset hypertension that is exacerbated in pregnancy. All steroids, including progesterone, that display antagonist properties when bound to the wild-type MR are able to activate the mutant receptor (MR_{L810}). These findings suggest that progesterone may contribute to the dramatic aggravation of hypertension in MR_{L810} carriers during pregnancy. However, the steroid(s) responsible for hypertension in MR_{L810} carriers (men and nonpregnant women) has not yet been identified. Here we show that cortisone and 11-dehydrocorticosterone, the main cortisol and corticosterone metabolites produced in the distal nephron, where sodium reabsorption stimulated by aldosterone takes place, bind with high affinity to MR_{L810}. The potency with which cortisone and 11-dehydrocorticosterone bind to the mutant MR contrasts sharply with their low wild-type MR-binding capacity. In addition, cotransfection assays demonstrate that cortisone and 11-dehydrocorticosterone are potent activators of the MR_{L810} *trans*-activation function. Because the plasma concentration of cortisol in humans is about 30-fold higher than that of corticosterone, these findings strongly suggest that cortisone is one of the endogenous steroids responsible for early-onset hypertension in men and nonpregnant women carrying the MR_{L810} mutation. (*Endocrinology* 144: 528–533, 2003)

UNDER NORMAL conditions, aldosterone binds with high affinity to the mineralocorticoid receptor (MR) to stimulate renal sodium reabsorption. In response to aldosterone, MR modulates transcription, and this ultimately enhances the transport of sodium from the tubular lumen to the basolateral side of the principal cells of the collecting duct (1, 2). Screening for MR among 75 hypertensive patients, many with low plasma renin and/or low serum aldosterone levels, identified a missense mutation in exon 6 of the MR in a young boy, resulting in the substitution of leucine for serine (S810L) at codon 810 (MR_{L810}) (3). Examination of this patient's family revealed that all MR_{L810} carriers had developed hypertension before they were 20 yr old, a rare trait in the general population, whereas the members of the family who did not express the mutant MR had normal blood pressure (3). Rather intriguingly, the women harboring this mutation experienced a dramatic exacerbation of hypertension during pregnancy.

In vitro experiments have revealed that aldosterone activates both wild-type MR (MR_{WT}) and MR_{L810} (3). Furthermore, all steroids, including progesterone, that displayed antagonist properties when bound to MR_{WT} were also able to activate MR_{L810}. As the levels of plasma progesterone normally increase about 100-fold during pregnancy, Geller *et al.* (3) have postulated that this hormone may contribute to the dramatic exacerbation of hypertension in pregnant MR_{L810} carriers. The steroid(s) responsible for early-onset hypertension in MR_{L810} carriers (men and nonpregnant women) has still not been identified.

Cortisol, which is 100- to 1000-fold more abundant than aldosterone in plasma, binds to MR with the same affinity as aldosterone (4, 5). In the collecting duct, the preferential accessibility of aldosterone to MR is ensured by 11β-hydroxysteroid dehydrogenase type 2, an enzyme that metabolizes cortisol and corticosterone to form inactive cortisone and 11-dehydrocorticosterone (6, 7). Because the determinants for the ligand-induced activation of MR_{L810} are distinct from those for MR_{WT}, we investigated the ability of cortisone and 11-dehydrocorticosterone to bind to MR_{L810} and activate its *trans*-activation function. The findings of steroid binding studies and steroid-induced *trans*-activation measurements provide evidence of the specific binding of cortisone and 11-dehydrocorticosterone to MR_{L810} and of their abilities to activate the mutant MR. Because the plasma level of cortisol in men is about 30-fold higher than the plasma level of corticosterone (8), these findings also suggested that cortisone could be involved in the development of hypertension in young men and nonpregnant women harboring the S810L mutation.

Materials and Methods

Plasmids and construction

The expression plasmid pchMR contains the entire coding sequence of MR_{WT} (9). Plasmid pchMR$_{L810}$, encoding for MR_{L810}, was constructed from pchMR using the QuikChange procedure from Stratagene (Amsterdam, The Netherlands). The *Bpu*1102I-*Afl*II fragment of pchMR$_{L810}$ was subcloned into pchMR, after being sequenced to confirm that there was no other mutation in the sequence. Plasmid pFC31Luc contains the mouse mammary tumor virus (MMTV) promoter that drives the luciferase gene (10), and plasmid TAT3-TATA-Luc contains a trimerized hormone response element fused to the alcohol dehydrogenase minimal

Abbreviations: MMTV, Mouse mammary tumor virus; MR, mineralocorticoid receptor; MR_{WT}, wild-type mineralocorticoid receptor.

promoter driving luciferase (11). The pSVβ vector was from CLONTECH Laboratories, Inc. (Saint Quentin en Yvelines, France).

In vitro hormone binding assay

MR_{WT} and MR_{L810} were expressed *in vitro* using the T7-coupled rabbit reticulocyte lysate system from Promega Corp. (Charbonnieres, France). Lysates containing MR_{WT} or MR_{L810} were diluted 2-fold with TEGWD buffer [20 mM sodium tungstate and 1 mM dithiothreitol in 20 mM Tris-HCl, 1 mM EDTA, and 10% glycerol, pH 7.4 (TEG)], and incubated with 10 nM [^{3}H]aldosterone (Amersham Pharmacia Biotech, Les Ulis, France) with or without unlabeled steroids for 30 min at 20 C. Bound and free steroids were separated by the dextran-charcoal method: 25 μl lysate were stirred for 5 min with 50 μl 4% Norit A and 0.4% dextran T-70 in TEG buffer and centrifuged at 4500 $\times$ *g* for 5 min at 4 C. Bound steroid was measured by counting the radioactivity of the supernatant.

Kinetic experiments

MR_{WT} and MR_{L810} were expressed *in vitro* using the T7-coupled rabbit reticulocyte lysate system. The lysate was diluted 2-fold with ice-cold TEGWD buffer and then incubated with 10 nM [^{3}H]aldosterone or [^{3}H]cortisol for 30 min at 20 C. One half of the labeled lysate was kept at 20 C to measure the stability of the [^{3}H]steroid-MR complexes, and the other was incubated with 10 μM of the corresponding unlabeled steroid for various periods. Bound and free steroids were separated with dextran-charcoal. Parallel incubations containing [^{3}H]steroid plus a 1000-fold excess of unlabeled steroid were used to calculate the nonspecific binding. Results were corrected for receptor stability and expressed as a percentage of the binding measured at time zero.

Cell culture and transfection

COS-7 cells were cultured in DMEM (Life Technologies, Inc.-BRL, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2. Cells were maintained in the medium supplemented with 10% charcoal-stripped fetal calf serum for 4 h before and then throughout the transfection procedure. Cells were transfected by the phosphate calcium precipitation method (Profection, Promega Corp.). The phosphate solution, prepared for six-well trays, contained 5 μg of one of the receptor expression vectors (pchMR or pchMR_{L810}), 10 μg pFC31Luc or TAT3-TATA-Luc, and 2 μg pSVβ. The steroids to be tested were added to the cells 12 h after transfection, and the incubation was continued for an additional 24 h. Cell extracts were then prepared and assayed for luciferase (12) and β-galactosidase activities (13). To standardize the transfection efficiency, the relative light units obtained in the luciferase assay were divided by the OD obtained in the β-galactosidase assay.

Statistical analysis

Values are the mean ± SEM from at least three separate experiments. Statistical differences between groups were analyzed by *t* test. $P < 0.05$ was considered significant.

Results

MR_{L810} and MR_{WT} were expressed *in vitro* and tested for the ability to bind cortisone and 11-dehydrocorticosterone. As these two steroids were available as unlabeled molecules only, their abilities to inhibit [^{3}H]aldosterone binding were measured in competition experiments. MR_{L810} or MR_{WT} was incubated with 5 nM [^{3}H]aldosterone alone or with 1 μM aldosterone, cortisol, cortisone, corticosterone, or 11-dehydrocorticosterone. About 80% of the [^{3}H]aldosterone bound to MR_{L810} or MR_{WT} was displaced by a 200-fold excess of aldosterone, cortisol, or corticosterone (Fig. 1A), indicating that these hormones bind to MR_{WT} and to MR_{L810}. Cortisone did not displace the [^{3}H]aldosterone bound to MR_{WT}, but did inhibit the binding of [^{3}H]aldosterone to MR_{L810}. 11-Dehy-

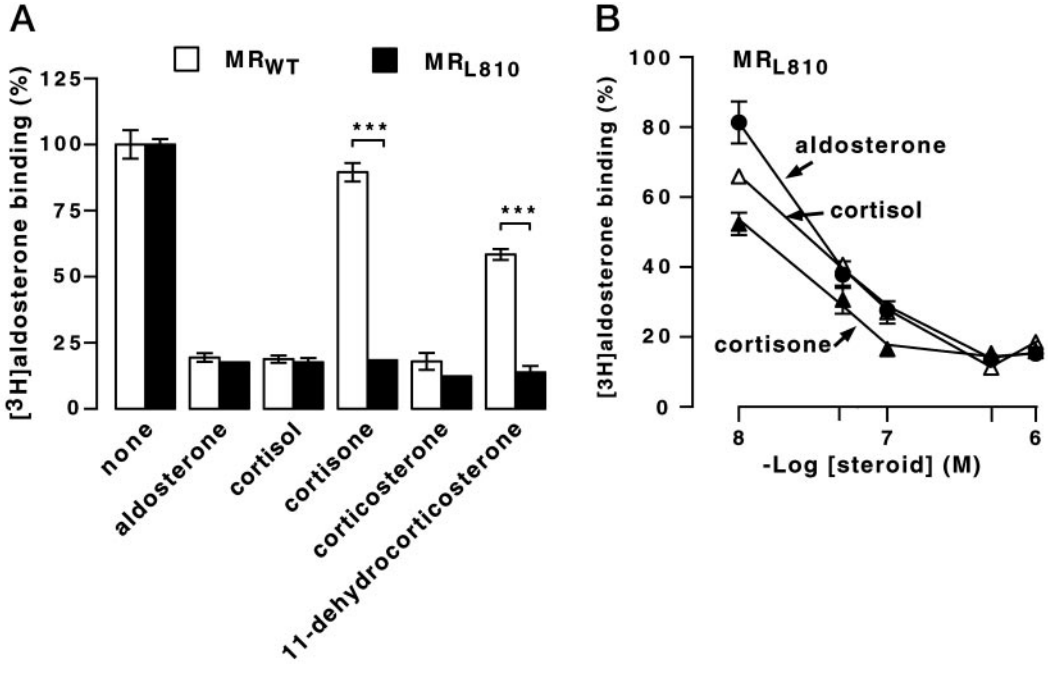

FIG. 1. Effects of steroids on the binding of tritiated aldosterone to MR_{WT} and mutant MR_{L810}. A, MR_{WT} and MR_{L810} were synthesized *in vitro* in rabbit reticulocyte lysate and incubated with 5 nM [^{3}H]aldosterone with or without 1 μM unlabeled aldosterone, cortisol, cortisone, corticosterone, and 11-dehydrocortisone for 2 h at 4 C. B, MR_{L810} expressed in rabbit reticulocyte lysate was incubated with 5 nM [^{3}H]aldosterone in the absence (100%) or presence of various concentrations of aldosterone, cortisol, or cortisone for 2 h at 4 C. Bound and unbound steroids were separated using the charcoal-dextran method. Results are expressed as a percentage of the specific [^{3}H]aldosterone binding measured in the absence of any competitor. Each *point* is the mean ± SEM of three separate experiments. ***, $P < 0.001$

drocorticosterone displaced by 40% and 80% the [^{3}H]aldosterone bound to MR_{WT} and MR_{L810}, respectively (Fig. 1A). The range of concentrations of the corticosteroids tested widely differs in humans. Table 1 summarizes the plasma levels of aldosterone, cortisol, cortisone, and corticosterone in men. It appears that cortisol is about 1000-fold more abundant than aldosterone in plasma. The prevalence of cortisol over corticosterone in plasma led us to further analyze the abilities of various concentrations of cortisol and cortisone to inhibit [^{3}H]aldosterone to both MR_{WT} and MR_{L810}. The dose-response curve depicted in Fig. 1B shows that cortisone was almost efficient as aldosterone and cortisol in displacing [^{3}H]aldosterone from MR_{L810}. This finding demonstrates the high affinity with which cortisone binds to MR_{L810}.

It has been reported that the stability of the steroid-receptor complexes is highly dependent upon the nature of the steroid (5). We therefore investigated whether the stability of the steroid-receptor complexes could be altered by the nature of amino acid residue at the 810 position of the MR. The half-life times ($t_{1/2}$) of steroid-MR_{WT} and steroid-MR_{L810} complexes were calculated from dissociation kinetics studies (Fig. 2). Aldosterone dissociated more slowly from MR_{L810} ($t_{1/2}$ = 420 min) than from MR_{WT} ($t_{1/2}$ = 90 min; Fig. 2). Cortisol also dissociated more slowly from MR_{L810} ($t_{1/2}$ = 120 min) than from MR_{WT} ($t_{1/2}$ = 10 min; Fig. 2). Figure 2 also shows that the dissociation kinetics were slower for the aldosterone-MR_{L810} complex ($t_{1/2}$ = 420 min) than for the cortisol-MR_{L810} complex ($t_{1/2}$ = 120 min). Similar differences in the dissociation kinetics and $t_{1/2}$ were observed with aldosterone- and cortisol-MR_{WT} complexes (Fig. 2). Despite the fact that S810L mutation has improved the stability of the human MR-steroid complexes, which suggests a change in receptor conformation, aldosterone-MR complexes still remain more stable than cortisol-MR complexes (Fig. 2).

TABLE 1. Plasma concentration of corticosteroids in humans

	Plasma concentration (nmol/liter)
Aldosterone	0.35
Cortisol	400
Cortisone	72
Corticosterone	12

The mean values reported in the table are from Ref. 8.

The *trans*-activation activity of MR_{L810} and MR_{WT} in response to steroids was then examined in *cis-trans* cotransfection assays. COS-7 cells were transiently transfected with pchMR_{L810} or pchMR_{WT} plus a reporter gene containing luciferase under the control of the MR-sensitive MMTV promoter (pFC31Luc). The transfected cells were incubated with 10 nM of the steroid to be tested. Aldosterone, cortisol, and corticosterone produced maximum activation of both MR_{WT} and MR_{L810} (Fig. 3A). Progesterone had low MR_{WT}-activating efficiency, but, like aldosterone, acted as a potent activator of MR_{L810} (Fig. 3A). Cortisone was unable to activate MR_{WT}, but activated MR_{L810} to about 75% the level of aldosterone-induced MR_{L810} activity (Fig. 3A). 11-Dehydrocorticosterone had a low MR_{WT}-activating efficiency, but maximally activated MR_{L810} (Fig. 3A). Additional transfection assays were performed using the TAT3-TATA-Luc promoter. In this case, 10^{-9} M cortisone, which slightly activated MR_{WT} (25% of the aldosterone-induced MR_{WT} activity), activated MR_{L810} to almost the same extent as aldosterone (Fig. 3A). These results indicated that the agonist activity of cortisone is related to the S810L mutation independently of the promoter used. The dose-response curves of MR_{WT} and MR_{L810} *trans*-activation activity performed with the MMTV promoter also

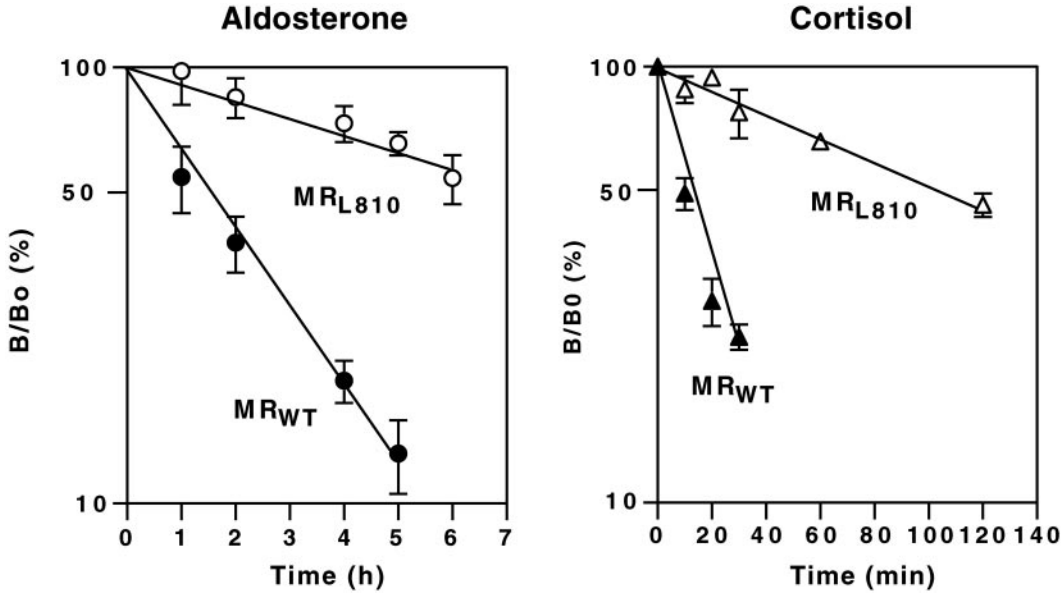

FIG. 2. Dissociation of aldosterone from MR_{WT} and MR_{L810}. MR_{WT} (*black symbols*) and MR_{L810} (*open symbols*) were produced by translation *in vitro* and incubated with 10 nM [^{3}H]aldosterone (*circles*) or [^{3}H]cortisol (*triangles*) for 30 min at 20 C. The end of this incubation period was taken as time zero for kinetic analysis. An aliquot was kept at 20 C to measure the stability of steroid-receptor complexes, and another aliquot was incubated with unlabeled aldosterone or cortisol (10^{-6} M). Bound and free steroids were separated by the dextran-charcoal method described in *Materials and Methods*. Nonspecific binding was measured in parallel for each time tested. Results were corrected for receptor stability and expressed as a percentage of the binding measured at time zero. Values are the mean ± SEM of three experiments.

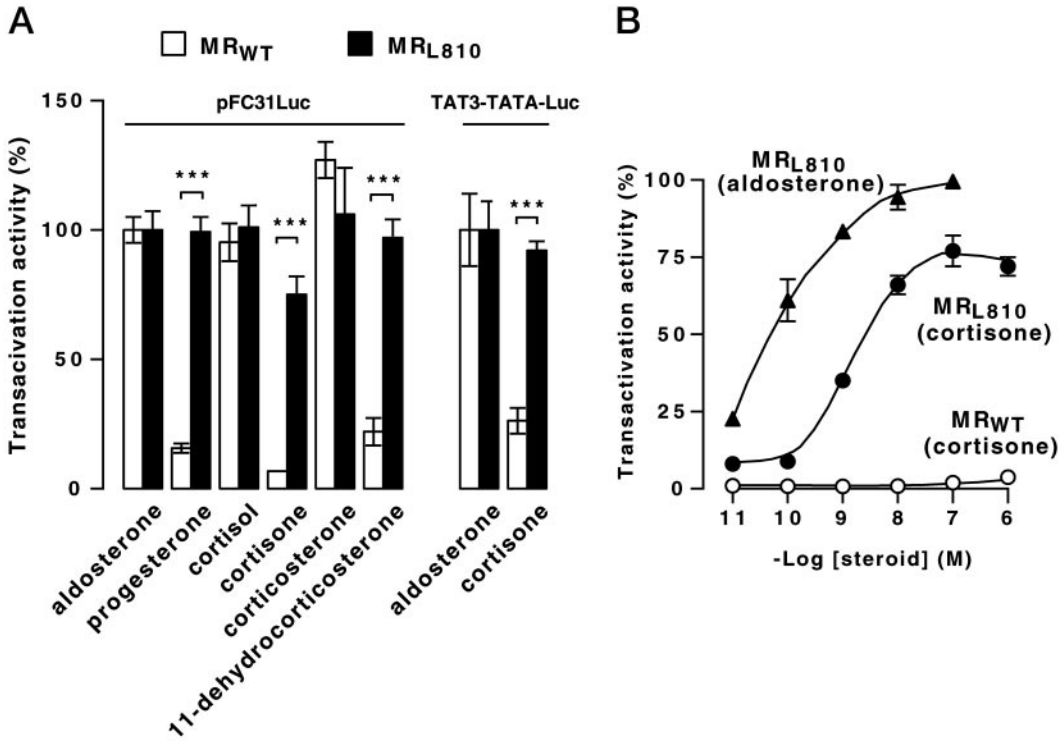

FIG. 3. *Trans*-activation activity of MR_{WT} and MR_{L810} in response to steroids. A and B, COS-7 cells were transfected with pchMR$_{WT}$ or pchMR$_{L810}$ and pFC31Luc or TAT3-TATA-Luc as reporter plasmids, and a β-galactosidase internal reporter was used to correct for transfection efficiency. A, Before harvesting, the cells were treated for 24 h with 10^{-8} M aldosterone, progesterone, cortisol, cortisone, corticosterone, or 11-dehydrocorticosterone (using pFC31Luc as reporter plasmid) or with 10^{-9} M aldosterone or cortisone (using TAT3-TATA-Luc as reporter plasmid). B, Before harvesting, the cells transfected with pFC31Luc were treated for 24 h with increasing concentrations of aldosterone or cortisone. *Trans*-activation was determined by luciferase activity, normalized *vs.* the internal β-galactosidase control, and expressed as a percentage of MR_{WT} activity measured in the presence 10^{-8} M aldosterone. Each *point* is the mean ± SEM of three separate experiments. ***, $P < 0.001$.

showed that cortisone had no effect on MR_{WT} activity, and that the concentration of cortisone required to produce half-maximum MR_{L810} *trans*-activation activity was higher (5×10^{-9} M) than that of aldosterone (5×10^{-11} M; Fig. 3B).

Discussion

The findings show that cortisone, the main metabolite of cortisol in the kidney, activates mutant MR_{L810}, suggesting that cortisone is primarily involved in the early development of hypertension in patients harboring this mutation.

Using cotransfection assays, Geller *et al.* (3) have shown that MR_{L810} displays a constitutive activity not detected for MR_{WT}, and these researchers point out that this activity, which they observed without adding any steroid, may play a role in the onset of hypertension in patients harboring mutant MR_{L810}. However, such constitutive activity accounts for only 20% of the maximum aldosterone-induced activity, suggesting that an endogenous steroid ligand is necessary for maximum MR_{L810} activation to promote hypertension. In their search for the endogenous steroid ligand activator, Geller *et al.* (3) tested the abilities of various steroids to activate MR_{L810}. Neither estradiol nor testosterone, two steroids with a 17β-hydroxyl group, activated MR_{L810} or MR_{WT} (3). In contrast, steroids with a 21-hydroxyl group, such as aldosterone and cortisol, activated MR_{WT} and MR_{L810} to the same extent (Ref. 3 and the present study). Steroids without the 21-hydroxyl group, which display antagonist properties when bound to the MR_{WT}, are also able to activate MR_{L810} (3). This is the case for the antihypertensive drug spironolactone (a synthetic steroid with 17γ-lactone) and progesterone (Ref. 3 and the present study). The facts that 17α-hydroxyprogesterone is able to activate MR_{L810} and the plasma level of this progesterone derivative is the same as that of aldosterone raised the possibility that 17α-hydroxyprogesterone may contribute to hypertension in men (3). In the present study we investigated the possibility that one of the steroid metabolites produced in the principal cells of the collecting duct could produce agonist effects at MR_{L810}, but not at MR_{WT}. We found that this was indeed the case for cortisone and 11-dehydrocorticosterone. The 11-dehydrometabolites of cortisol and corticosterone have very low agonist and antagonist activities at high concentrations when bound to MR_{WT} (14, 15). In sharp contrast, we found that cortisone and 11-dehydrocorticosterone bind and activate MR_{L810}. Under normal conditions, cortisol and corticosterone are metabolized by 11β-hydroxysteroid dehydrogenase type 2 to form the almost inactive substance, cortisone and 11-dehydrocorticosterone, respectively, allowing aldosterone to occupy the MR, and this is responsible for the fine hormonal regulation of sodium absorption in the distal nephron (6, 7, 15). Because plasma levels of corticosterone are much lower than those of cortisol, it seems unlikely that 11-dehydrocortisosterone will

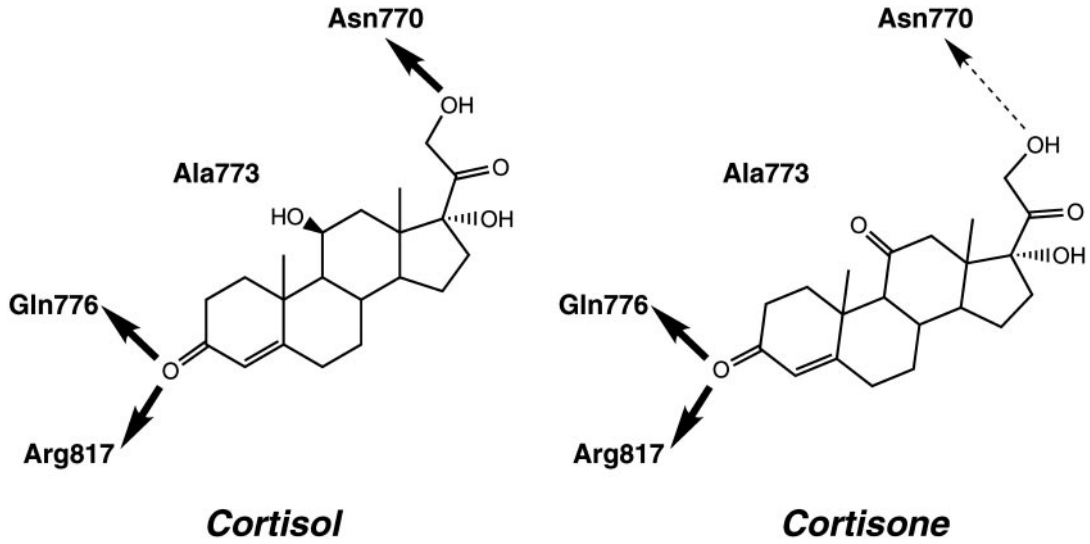

FIG. 4. Schematic representation of the amino acid residues of MR_{WT} involved in the anchoring of cortisol and cortisone. The *black arrows* represent the hydrogen bonds between steroids and amino acid residues of the ligand-binding domain of MR_{WT}. The Ala^{773} facing the 11-ketone of cortisone impairs its positioning within the ligand binding pocket of MR_{WT}, leading to a weak contact between the 21-hydroxyl group of cortisone and Asn^{770} (*dashed arrow*).

have any effect on MR_{L810} activation *in vivo*. Cortisol, the main plasma corticosteroid in humans, is metabolized into cortisone in collecting duct cells (15). Cortisone, inactive on MR_{WT}, becomes active on MR_{L810}. The dose-response curve of MR_{L810} *trans*-activation activity in response to cortisone suggests that the concentration of circulating cortisone is sufficient (~70 nM) to activate the mutant MR. Thus, in MR_{L810} carriers, the occupancy of MR by cortisone should lead to permanent activation of the mutant MR.

The agonist activity of aldosterone at the hMR has been attributed to the capacity of its 21-hydroxyl group to form a strong hydrogen bond with Asn^{770}, a residue of the H3 helix (9). The presence of a hydroxyl group in the C_{11} position facing alanine 773 (A773) of the H3 helix interferes with the positioning of cortisol within the ligand-binding pocket of the hMR (9, 15, 16). As a result, the contact between the 21-hydroxyl of cortisol and Asn^{770} is weakened, and the MR_{WT}-activating potential of cortisol is lower than that of aldosterone (9). The accommodation of cortisone in the ligand binding pocket is even more difficult, making contact between Asn^{770} and the 21-hydroxyl group almost impossible. This hypothesis is illustrated in Fig. 4. The lack of contact between Asn^{770} and the 21-hydroxyl group is compatible with the antagonist activity of cortisone, as has been observed for progesterone and spirolactone (9).

The exact mechanism of MR_{L810} activation remains unknown. It has been proposed that the agonist activity of MR_{L810} when bound to steroids with no 21-hydroxyl group, such as progesterone, depends on contacts between the H3 and H5 helixes via the Leu^{810} and Ala^{773} residues (3). Cortisone can also activate MR_{L810} through similar contacts. Another possible explanation, and the two are not mutually exclusive, is that the S810L mutation could modify the conformation of the mutant receptor, making it possible to accommodate cortisone within the ligand binding pocket of the mutant MR. In this case, the contact between the 21-hydroxyl group of cortisone and Asn^{770}, which is responsible for the activation of MR_{WT} by aldosterone (9), may make the cortisone-dependent agonist activity of mutant MR_{L810} possible. The question also arises of whether the high stability of the steroid-MR_{L810} complex contributes to permanent activation of the mutant receptor. Although this possibility cannot be completely ruled out, Geller *et al.* (3) showed that MR_{WT} and MR_{L810} displayed the same sensitivity to aldosterone.

The activating S810L mutation that has been found to date in only one family (3) has brought new insights to the understanding of MR agonism mechanisms. In this study we show that cortisone, which is normally an inefficient activator of MR_{WT}, is able to activate MR_{L810}. Thus, it is reasonable to infer that the early onset of hypertension in MR_{L810} carriers could be attributable to a permanent increase in renal sodium reabsorption due to activation of MR_{L810} by cortisone.

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