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Cortisol Metabolism in the Bolivian Squirrel Monkey (*Saimiri boliviensis boliviensis*)

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New World squirrel monkeys (*Saimiri* spp.) have high circulating cortisol levels but normal electrolytes and blood pressures. The goal of the present study was to gain insight into adaptive mechanisms used by Bolivian squirrel monkeys to minimize the effects of high cortisol on mineralocorticoid receptor (MR) activity and electrolyte and water balance. Aldosterone levels in serum from 10 squirrel monkeys were 17.7 ± 3.4 ng/dl (normal range in humans, 4 to 31 ng/dl), suggesting that squirrel monkeys do not exhibit a compensatory increase in aldosterone. The squirrel monkey MR was cloned and expressed in COS-7 cells and found to have similar responsiveness to cortisol and aldosterone as human MR, suggesting that squirrel monkey MR is not inherently less responsive to cortisol. To determine whether altered metabolism of cortisol might contribute to MR protection in squirrel monkeys, serum and urinary cortisol and cortisone were measured, and a comprehensive urinary corticosteroid metabolite profile was performed in samples from anesthetized and awake squirrel monkeys. The levels of cortisone exceeded those of cortisol in serum and urine, suggesting increased peripheral 11β-hydroxysteroid dehydrogenase 2 activity in squirrel monkeys. In addition, a significant fraction (approximately 20%) of total corticosteroids excreted in the urine of squirrel monkeys appeared as 6β-hydroxycortisol, compared with that in man (1%). Therefore, changes in cortisol metabolism likely contribute to adaptive mechanisms used by Bolivian squirrel monkeys to minimize effects of high cortisol.

Abbreviations: EC_{50} value, concentration of ligand that produces 50% of the maximum response; MR, mineralocorticoid receptor

Glucocorticoids play an essential role in many physiologic and biochemical processes, influencing the nervous system, cardiovascular system, and connective and lymphoid tissue as well as endocrine tissues. Disruption of glucocorticoid action is incompatible with life.[11] In humans and nonhuman primates, the principal circulating glucocorticoid is cortisol that is variably bound to serum proteins, especially corticosteroid-binding globulin.[25] Circulating cortisol levels are maintained not only by secretion from the adrenal cortex but also by metabolism in peripheral tissues.[46] Key pathways involved in the metabolism of cortisol are shown in Figure 1. One of the most important pathways for the metabolism of cortisol is catalyzed by the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2).[43,50] This enzyme is responsible for converting cortisol to cortisone, which is inactive.

Cortisol circulates in humans at a level approximately 1000-fold higher than aldosterone. Therefore, it was a dilemma when the mineralocorticoid receptor (MR) was first cloned and found to exhibit the same affinity in vitro for cortisol and aldosterone.[2] It was quickly recognized that the expression of 11β-HSD2 at the site of MR allows for efficient inactivation of cortisol. This process enables aldosterone to bind to its receptor in vivo, which leads to sodium resorption from and potassium excretion into the tubular lumen of the nephron.[15,21] A number of studies have shown that pharmacologic inhibition of 11β-HSD2 or mutations in the 11β-HSD2 gene (*HSD11B2*) lead to inappropriate activation of the MR by circulating cortisol with resultant hypertension and hypokalemia.[33,43,50] Patients with inappropriately elevated serum cortisol, due to Cushing's disease from excess adrenocorticotropin secretion or an adrenal tumor, also may exhibit hypertension.[14,46] Although there is considerable capacity in vivo for 11β-HSD2 to convert cortisol to cortisone, saturation of 11β-HSD2 can result in hypertension.[30,48]

Some animal species of the Americas have high circulating cor-

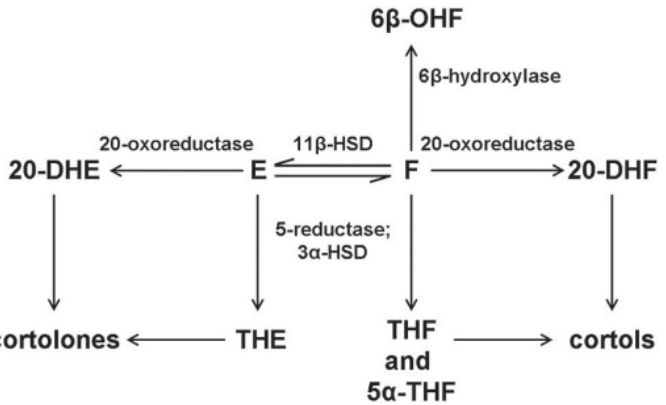

Figure 1. The principal pathways of cortisol metabolism in vivo. Interconversion of cortisol (F) and cortisone (E) is catalyzed by 2 isoenzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD), 11β-HSD1 and 11β-HSD2. Cortisol is converted to 6β-hydroxycortisol (6β-OHF) by 6β-hydroxylase. Cortisol and cortisone are converted to 20-dihydrocortisol (20-DHF) and 20-dihydrocortisone (20-DHE) by 20-oxoreductase, respectively, and are converted by 5-reductase and 3β-hydroxysteroid dehydrogenase (3β-HSD), respectively, to tetrahydrocortisol (THF), 5β-THF, and tetrahydrocortisone (THE).

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tisol levels, secondary to the expression of glucocorticoid receptors with low affinity.[9,19,35] One of the most striking examples is the squirrel monkey (*Saimiri sp.*), in which free plasma cortisol levels are approximately 100-fold higher than those in humans.[9,25] However, squirrel monkeys maintain normal plasma electrolytes and blood pressure,[6,10,41] suggesting that squirrel monkeys possess adaptive mechanisms to minimize the effects of high cortisol on electrolyte balance. The nature of these adaptations is unknown. To gain insight into adaptive mechanisms in squirrel monkeys, we have: (1) measured the circulating levels of cortisol and aldosterone in squirrel monkeys; (2) cloned the squirrel monkey MR and compared its activity to human MR in response to cortisol and aldosterone; (3) identified predominant pathways of cortisol metabolism in the squirrel monkey.

Materials and Methods

Materials. Culture medium was obtained from Life Technologies (Grand Island, NY). Defined and charcoal- and dextran-treated fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). pRSVhMR and mouse mammary tumor virus promoter–luciferase reporter plasmids were provided by Dr RM Evans (Salk Institute, La Jolla, CA). Cortisol and aldosterone were obtained from Sigma (St Louis, MO) and Acros Organics (Morris Plains, NJ), respectively.

Subjects and social environment. Subjects were adult Bolivian squirrel monkeys (*Saimiri boliviensis boliviensis*) that were housed indoors (22 °C) in laboratory breeding groups. Animals were exposed to a natural light:dark cycle that tracked local sunrise and sunset. Animals were fed a diet of Purina New World primate diet 5040 (PMI Nutrition International, St Louis, MO) and seasonal produce. Peanuts and Prima-Treats (Bio-Serv, Frenchtown, NJ) were offered as environmental enrichment. All experiments were approved by the Institutional Animal Care and Use Committee of the University of South Alabama.

Blood and urine sampling was performed between the hours of 08:00 and 10:00 h. Blood samples from unanesthetized animals were obtained by femoral venipuncture within 2 min of capture. Blood samples for measurement of total serum cortisol and aldosterone levels as well as electrolytes (Na^+ and K^+) were obtained from female squirrel monkeys during late February and March. Serum was separated by centrifugation at $1200 \times g$ for 20 min and stored at –80 °C until assayed. Blood samples for measurement of cortisol and cortisone were obtained from 4 awake (August) or 6 anesthetized (September to October) male squirrel monkeys, and serum was collected as described earlier. Anesthesia was achieved with 10 mg/kg ketamine and 3 mg/kg xylazine subcutaneously.

In addition, urine samples for determination of free cortisol, cortisone, and 6β-hydroxycortisol were collected from 6 anesthetized, male squirrel monkeys (September) by gentle palpation of the bladder and collection in 15-ml conical tubes. The samples were centrifuged at $1200 \times g$ for 20 min to remove particulate matter. Aliquots were removed for determination of osmolality by using a freezing point depression osmometer (Advanced Micro Osmometer, Norwood, MA) prior to freezing at –80 °C. Urine samples for determination of urinary cortisol metabolites were collected in April from 1 male and 2 female squirrel monkeys housed separately in metabolic cages during the collection period.

Assays. Serum cortisol and aldosterone were measured at the University of South Alabama Health Services Foundation Laboratories (Mobile, AL) by using an Immulite 2000 Advanced Immunoassay System (Diagnostic Products, Los Angeles, CA). Electrolytes were measured on a Dimension RxL Analyzer (Dade Behring, Deerfield, IL). Serum and urinary cortisol, cortisone, and 6β-hydroxycortisol were measured by liquid chromatography–tandem mass spectrometry, as described previously.[45] Comprehensive urinary corticosteroid metabolite profiles were analyzed using gas chromatography–mass spectrometry.[30,40]

cDNA cloning. Total RNA was isolated with RNA Stat-60 (Tel-Test B, Friendswood, TX) from kidneys from an adult, female Bolivian squirrel monkey obtained at necropsy through the Tissue and Biological Fluids Resource of the Center for Neotropical Primate Research and Resources (Mobile, AL). Two cDNA fragments of the Bolivian squirrel monkey MR were generated by reverse transcription–polymerase chain reaction (RT-PCR) using primers GAT GGA GAC CAA AGG CTA CCA C (sense; corresponding to nucleotides 3 through 24) and CAG GTG TTG GAA AGA TTG GTC TC (antisense; nucleotides 1641 through 1619) and primers GAT TGG TGC TCA AGG TAC AAT ATC (sense; nucleotides 1578 through 1601) and GGC AGT CAC TTC CGG TGG AAG (antisense; nucleotides 2957 through 2937) in the Guyanese squirrel monkey (*Saimiri sciureus*) MR cDNA (GenBank accession number, AF245224). RT-PCR was performed using a OneStep RT-PCR Kit (Qiagen, Valencia, CA). The full-length Bolivian squirrel monkey MR cDNA was amplified by PCR using primers GTC AAG CTT GGG ATG GAG ACC AAA GGC TAC (sense; *Hind*III site underlined) and TCA GTC GAC GGC AGT CAC TTC CGG TGG AAG (antisense; *Sal*I site underlined) and the 2 cDNA fragments as template. The full-length MR cDNA was cloned into *Hind*III- and *Sal*I-digested pRSV mammalian expression vector[2] to yield pRSVsmMR, and the insert was sequenced across both strands.

Full-length cDNA sequence also was obtained for owl monkey MR. A kidney was obtained from an adult, female owl monkey (*Aotus nancymaae*) at necropsy through the Tissue and Biological Fluids Resource of the Center for Neotropical Primate Research and Resources. The MR cDNA was amplified by RT-PCR as described, and the PCR product was sequenced across both strands. The amino acid sequences of the Bolivian squirrel monkey and owl monkey MRs were deduced from the nucleotide sequences.

Cell cultures. COS-7 cells were grown as monolayers in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin G, and 0.05 mg/ml streptomycin. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air. At 24 h prior to transfection, cells were transferred to the same medium supplemented with 10% charcoal- and dextran-treated fetal bovine serum. Cells were transfected with plasmids expressing human (pRSVhMR) or squirrel monkey MR (pRSVsmMR) and the mouse mammary tumor virus promoter–luciferase reporter vector (MMTV-luciferase) by using a modification of the method of Bodwell and others,[4] as described previously[13] except that each electroporation cuvette contained 6 μg MR plasmid, 10 μg MMTV-luciferase plasmid, and 1×10^7 cells. Electroporated cells were diluted in medium supplemented with 10% charcoal- and dextran-treated fetal bovine serum and plated in 60-mm dishes at a density of 1×10^6 cells/dish. After 18 h, medium was replaced and cells were treated with either aldosterone (1 pM to 1 μM) or cortisol (0.1 nM to 100 μM). After 24 h, cells were lysed and assayed for luciferase activity as described.[24] EC_{50} values (defined as the concentration of ligand that produces 50% of the maximum response) were obtained from the concentration–response curves.

Table 1. Concentrations of cortisol (F), aldosterone (Aldo), Na^+, and K^+ in the serum of 10 female Bolivian squirrel monkeys

Animal no.	F (μg/dl)	Aldo (ng/dl)	Na^+ (mEq/l)	K^+ (mEq/l)
1962	117	13.0	149	4.4
2432	233	39.1	148	4.5
2664	216	13.3	149	5.0
1519	320	27.6	139	5.2
2844	145	17.8	153	5.4
2808	157	18.9	154	4.4
90747	159	15.8	149	5.1
1372	234	4.4	148	4.5
1528	169	21.5	147	4.6
1855	126	5.4	147	4.5

Blood was collected during late February and early March by femoral venipuncture within 2 min of capture. Serum was separated by centrifugation and assayed using a commercial immunoassay system. Human reference values: cortisol, 5 to 25 μg/dl; aldosterone, 4 to 31 ng/dl; Na^+, 140 to 148 mEq/l; and K^+, 3.6 to 5.2 mEq/l.

Results

Circulating aldosterone in squirrel monkeys. The overall goal of these studies was to gain insight into the adaptive mechanisms developed by squirrel monkeys to minimize the effects of high cortisol on electrolyte balance. The 1st question asked was whether aldosterone levels were elevated in squirrel monkeys, so that the relative ratio of cortisol to aldosterone did not differ from that in man or Old World primates. Previous studies, some published more than 20 y ago, indicated that squirrel monkeys have elevated circulating aldosterone levels.[6,10,41] Uncertainty regarding the specificity of aldosterone antibodies used in the immunoassays and the conditions under which the samples were obtained encouraged us to repeat these measurements in unanesthetized squirrel monkeys that were provided water and food ad libitum and only briefly restrained for blood drawing. Samples were obtained from 10 female Bolivian squirrel monkeys during late February and March and analyzed for concentrations of cortisol, aldosterone, Na^+, and K^+ (Table 1). There was animal-to-animal variability in the circulating levels of cortisol and aldosterone. However, when taken together, the serum levels of cortisol (mean ± standard error of the mean [SEM], 188 ± 21 μg/dl), Na^+ (148 ± 1 mEq/l), and K^+ (4.8 ± 0.1 mEq/l) in squirrel monkeys were similar to values previously reported in the literature.[6,9] Serum aldosterone levels in cohort monkeys (17.7 ± 3.4 ng/dl, n = 10) were lower than values previously reported for squirrel monkeys (range 30 to 125 ng/dl)[6,10,41] and well within the range reported for man (4 to 31 ng/dl). These results suggest that an increase in aldosterone secretion is not one of the mechanisms employed by squirrel monkeys to minimize the effects of high cortisol. Therefore, we turned our attention toward the MR and asked whether the squirrel monkey MR was differentially responsive to aldosterone and cortisol.

Molecular cloning of squirrel monkey MR. Previous studies suggested that the absence of mineralocorticoid excess syndrome in squirrel monkeys results from an alteration in the ability of cortisol to bind MR.[6,7] However, it is not clear whether this altered binding is an inherent characteristic of the squirrel monkey MR or whether hormone binding is influenced by the enzymatic milieu of squirrel monkey tissue extracts. To investigate this issue directly, we cloned and sequenced MR from Bolivian squirrel monkey kidney. The nucleotide and amino acid sequences of the Bolivian squirrel monkey MR (GenBank accession number, DQ072160) are 99.8% identical to those of the Guyanese squirrel monkey MR (GenBank accession number, AF245224) previously reported by Patel and others.[31] Differences between the Bolivian and Guyanese squirrel monkey MRs are found at position 292, where leucine is substituted for proline, and at position 673, where lysine is substituted for arginine (Figure 2). Compared with human MR (GenBank accession number, M16801), Bolivian and Guyanese squirrel monkey MRs share deletions of serine at position 118 and of glutamine at position 690. Otherwise, the squirrel monkey MRs exhibit 98% identity with human MR, although 75% of the differences represent marked changes in charge or polarity. For comparative purposes, we cloned and sequenced the MR from owl monkey, another New World primate with elevated serum cortisol.[9] Owl monkey MR is 99.2% identical with Bolivian and Guyanese squirrel monkey MRs (Figure 2) but does not share the deletions at positions 118 and 690. In this regard, the owl monkey MR is more similar to human MR, with which it is 98.5% identical overall. The owl monkey MR cDNA sequence has been deposited in GenBank (accession number, DQ143871).

Activity of squirrel monkey MR. The 2nd goal of this study was to determine whether the squirrel monkey MR is less responsive to cortisol than is human MR. Human and Bolivian squirrel monkey MRs were expressed in heterologous African green monkey COS-7 cells. COS-7 cells have been used extensively to test the activity of wild-type and mutant MRs.[16,44] We found that squirrel monkey and human MRs show similar activation profiles in response to cortisol in COS-7 cells (Figure 3A). The EC_{50}s for cortisol-mediated transactivation of squirrel monkey and human MRs were 4.0 ± 1.6 nM (mean ± SEM, n = 3) and 5.0 ± 2.4 nM (n = 3), respectively. Squirrel monkey and human MRs also showed similar activation by aldosterone (EC_{50}s of 77 ± 30 pM [n = 3] and 51 ± 20 pM [n = 3], respectively [Figure 3B]). Cortisol and aldosterone have identical affinities for human MR in binding studies.[2,22] But, in transactivation studies, such as in the COS-7 cells described here, aldosterone exhibits significantly higher potency than cortisol.[16,22] These results suggest that when expressed in heterologous COS-7 cells, squirrel monkey and human MR share similar responsiveness to aldosterone and cortisol. Thus, squirrel monkey MR is not inherently less responsive to cortisol than human MR, suggesting that other factors must be responsible for protecting MR from excessively high levels of cortisol in squirrel monkeys. We examined whether altered metabolism of cortisol might contribute to MR protection in the squirrel monkey.

Cortisol and cortisone in squirrel monkey serum and urine. We measured serum and urinary levels of free cortisol and cortisone in squirrel monkeys. The ratio between cortisol and cortisone has been used as a marker of 11β-HSD activity.[23,46] Cortisol and cortisone were measured by liquid chromatography–tandem mass spectroscopy first in the serum of 4 awake, male squirrel monkeys and later in the serum and urine from 6 male squirrel monkeys under anesthesia (Table 2). There was animal-to-animal variability in steroid hormone levels regardless of whether squirrel monkeys were awake or anesthetized. However, when taken together, serum levels of cortisol were 96 ± 14 μg/dl, whereas serum cortisone averaged 108 ± 13 μg/dl. The ratio of cortisol to cortisone in the sera of squirrel monkeys (0.93 ± 0.12) is strikingly lower than that of the sera of normal human subjects (3.0 to 7.3).[23,26,42] The serum levels of cortisol in squirrel monkeys sampled here between August and October were lower than values obtained in monkeys sampled in February and March (188 ± 21 μg/dl, Table 1). These results are consistent with previous studies that described seasonal variation in serum cortisol levels in both sexes of squirrel monkeys.[36,37]

We also measured the levels of cortisol and cortisone in urine from spot samples obtained from anesthetized monkeys. The ab-

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bsmMR    METKGYHSLP EGLDMERRWG QVSQAVEHSS LGSTERTDEN NYMEIVNVSC VSGAIPNNST QGSSKEKHEL LPCLQQDNNR  80
gsmMR    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  80
omMR     ---------- ---------- ----T----- ---------- ---------- ---------- ---------- ----------  80
humanMR  ---------- ---------- -------R-- --P------- ---------- ---------- -------Q-- ----------  80

bsmMR    PGILTSDIKT ELESKELSAT VAESMGLYMD SVRDADY*YE QQNQQRSMSP AKIYQNVEQL VKFYKENGHR PSTLSCVNRP  159
gsmMR    ---------- ---------- ---------- -------*-- ---------- ---------- ---------- ----------  159
omMR     ---------- ---------- ---------- -------S-- ---------- ---------- ---------- ----------  160
humanMR  ---------- ---------- ---------- -------S-- -----G---- ---------- -----G---- --------T-  160

bsmMR    LRSFMSDSVS SVNGGVMRAI VKSPIMCHEK SPSVCSPLNM TSSVCSPAGI NSVSSTTASF GSFPVHSPIT QGTPLTCSPN  239
gsmMR    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  239
omMR     --------G- ---------- ---------- ---------- ---------- ---------- ---------- ----------  240
humanMR  --------G- ---------- ---------- ---------- ---------- ---------- ---------- ----------  240

bsmMR    VENRGSRSHS PAHASNVGSP LSSPLSSMKS SISSPPSHCS VKSPVSSPNN VTLRSSVSSP ANINNSRCSV SSPSNTNNRS  319
gsmMR    ---------- ---------- ---------- ---------- ---------- --P------- ---------- ----------  319
omMR     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  320
humanMR  A--------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  320

bsmMR    TLSSPAASTV GSICSPVNNA FSYTASGTSA GSSTSRDVVP SPDTQEKGAQ EVPFPKTEEV ESAISNGVTG QLNIVQYIKP  399
gsmMR    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  399
omMR     ---------- ---------- ---------- ---------- ---------- --L------- ---------- ----------  400
humanMR  ---------- ---------- ---------- ----L----- ---------- ---------- ---------- ----------  400

bsmMR    EPDGAFSSSC LGGNSKINSD SPFSVPIKQE STKHSCSGTS FKGNPTVNPF PFMDGSYFSF MDDKDYYSLS GILGPPVPGF  479
gsmMR    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  479
omMR     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  480
humanMR  ---------- ---------- -S-------- ---------- ---------- ---------- ---------- ----------  480

bsmMR    DGTCEGSGFP VGIKQEPDDG SYYPEASIPS SAIVGVNSGG QSFHYRIGAQ GTISLSRSAR DQSFQHLSSF PPVNTLVESW  559
gsmMR    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  559
omMR     --N------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  560
humanMR  --N------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  560

bsmMR    KSHGDLSSRR SDGYPVLEYI PENVSSSTLR SVSTGSSRPS KICLVCGDEA SGCHYGVVTC GSCKVFFKRA VEGQHNYLCA  639
gsmMR    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  639
omMR     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  640
humanMR  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  640

bsmMR    GRNDCIIDKI RRKNCPACRL QKCLQAGMNL GARKSKKLGK LKGIHEEQP* QQQPPPPPPP PQSPEEGTTY IAPAKEPSVN  718
gsmMR    ---------- ---------- ---------- ---R------ ---------* ---------- ---------- ----------  718
omMR     ---------- ---------- ---------- ---------- ---------Q ---------- ---------- ----------  720
humanMR  ---------- ---------- ---------- ---------- ---------Q ---------- ---------- ----------  720

bsmMR    TALVPQLSAI SRALTPSPAM VLENIEPEVV YAGYDNSKPD TAENLLSTLN RLAGKQMIQV VKWAKVLPGF KNLPLEDQIT  798
gsmMR    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  798
omMR     --------T- ---------- I--------- ---------- ---------- ---------- ---------- ----------  800
humanMR  --------T- --------V- --------I- -----S---- ---------- ---------- ---------- ----------  800

bsmMR    LIQYSWMCLS SFALSWRSYK HTNSQFLYFA PDLVFNEEKM HQSAMYELCQ GMHQISLQFI RLQLTFEEYT IMKVLLLLST  878
gsmMR    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  878
omMR     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  880
humanMR  ---------- ---------- ---------- ---------- ---------- ---------V ---------- ----------  880

bsmMR    VPKDGLKSQA AFEEMRTNYI KELRKMVTKC PNNSGQSWQR FYQLTKLLDS MHDLVNDLLE FCFYTFRESQ ALKVEFPAML  958
gsmMR    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  958
omMR     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  960
humanMR  I--------- ---------- ---------- ---------- ---------- -----S---- ---------H ----------  960

bsmMR    VEIISDQLPK VESGNAKPLY FHRK  982
gsmMR    ---------- ---------- ----  982
omMR     ---------- ---------- ----  984
humanMR  ---------- ---------- ----  984
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Figure 2. Comparison of the amino acid sequences of the Bolivian squirrel monkey (*Saimiri boliviensis*), Guyanese squirrel monkey (*Saimiri sciureus*), owl monkey (*Aotus nancymaae*), and human MRs. Guyanese squirrel monkey (gsmMR) and human MR (humanMR) sequences were obtained from GenBank (accession numbers AF245224 and M16801, respectively). The Bolivian squirrel monkey MR (bsmMR) and owl monkey cDNA sequences have been deposited in GenBank (accession numbers DQ072160 and DQ143871, respectively). Identical amino acids are indicated with a hyphen. Asterisks indicate gaps.

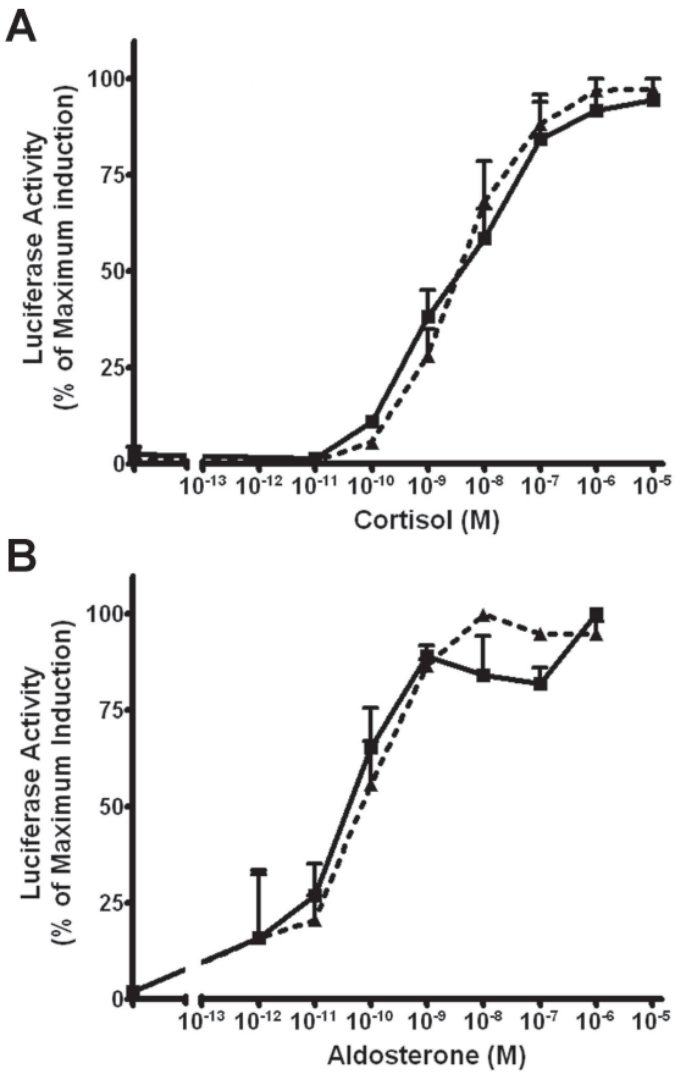

Figure 3. Transcriptional activity of the Bolivian squirrel monkey and human MRs in COS-7 cells. Squirrel monkey MR (smMR; solid line) and human MR (hMR; dotted line) plasmids were expressed in COS-7 cells with the MMTV-luciferase reporter plasmid. The medium was changed, and cells were treated with the indicated concentrations of either cortisol (A) or aldosterone (B). After 24 h, cells were collected for assay of luciferase activity.

solute values varied considerably between animals depending on the level of concentration of the urine, which was assessed by measuring osmolality. However, the ratio of cortisol to cortisone was relatively consistent among the animals (0.67 ± 0.11; Table 2). Overall, the ratio of cortisol to cortisone in urine of squirrel monkeys was similar to that measured in normal male human subjects (approximately 0.6).[30] Although these results have yielded information on 11β-HSD activity in squirrel monkeys, they do not provide insight into other pathways of cortisol metabolism.

Pathways of cortisol metabolism in squirrel monkeys. To gain a broader understanding of cortisol metabolism in squirrel monkeys, a comprehensive urinary corticosteroid metabolite profile was performed on samples from 2 female (numbers 1837 and 1447) and 1 male (1042) Bolivian squirrel monkey by using gas chromatography–mass spectroscopy. Samples were collected from unrestrained animals briefly housed in metabolic cages. The data are presented for each animal individually, expressed

Table 2. Free concentrations of cortisol (F) and cortisone (E) in the serum and urine of male Bolivian squirrel monkeys

Animal no.		F (μg/dl)	E (μg/dl)	F/E ratio
Serum (August, unanesthetized)				
3014		137	132	1.04
2569		31	93	0.33
91011		68	100	0.68
2212		69	45	1.53
Serum (September to October, anesthetized)				
2212		98	80	1.23
90848		64	77	0.83
2439		104	166	0.63
91014		159	127	1.25
2569		81	93	0.87
91011		148	169	0.88
Urine (September to October, anesthetized)				
	mOsm/l			
2212	567	46	84	0.55
90848	572	107	95	1.13
2439	499	99	152	0.65
91014	190	77	129	0.60
2569	130	38	86	0.44
91011	320	86	138	0.62

Serum was obtained from 4 unanesthetized squirrel monkeys (August), and serum and urine were obtained from 6 animals under ketamine and xylazine anesthesia (September to October). Human reference values: serum cortisol, 3.3 to 24.6 μg/dl; serum cortisone, 0.8 to 2.7 μg/dl.[23,26]

for each metabolite as a percentage of the total corticosteroid products excreted (Table 3). As previously reported,[38] squirrel monkeys excrete large amounts of free cortisol and cortisone but very little tetrahydrocortisol or tetrahydrocortisone, compared with humans. Another striking difference in metabolite excretion between squirrel monkeys and humans involves 6β-hydroxycortisol. Compared with the fraction of total corticosteroids normally excreted as 6β-hydroxycortisol in humans (approximately 1%), almost 20% of the total corticosteroids excreted in squirrel monkey urine appeared as 6β-hydroxycortisol (Table 3). A similarly high fraction of 6β-hydroxycortisol also occurred in the urine of owl monkeys (data not shown).

However, 6β-hydroxycortisol has not been found to be an important cortisol metabolite in all New World primates. Although 6β-hydroxycortisol is a predominant metabolite in capuchin monkeys (*Cebus albifrons*),[3] Shackleton found no evidence of 6β-hydroxycortisol excretion in common marmosets (*Callithrix jacchus*).[39] Furthermore, Setchell and colleagues did not report that 6β-hydroxycortisol was present in the urine of Guyanese squirrel monkeys (*Saimiri sciureus*).[38] These reports prompted us to expand our initial findings obtained by gas chromatography–mass spectroscopy of a small number of samples to analysis of a larger cohort of urine samples from Bolivian squirrel monkeys by using liquid chromatography–tandem mass spectroscopy (Table 4). Using this method, we confirmed that 6β-hydroxycortisol is a predominant cortisol metabolite in the urine of Bolivian squirrel monkeys, being excreted at a level that was approximately 80% (77% ± 13%) that of cortisol. The ratio of cortisol to cortisone in the urine of this group (0.59 ± 0.07) was similar to that observed in the other cohort sampled (0.67 ± 0.11, Table 2).

Discussion

The goal of these studies was to gain insight into the adaptive mechanisms of squirrel monkeys that minimize effects of extremely high cortisol levels on electrolyte balance and other

Table 3. Conjugated and unconjugated urinary corticosteroid metabolites in Bolivian squirrel monkeys[a]

	Animal no.				
	1837	1447	1042	Mean ± SEM	Human[b]
Cortisol	28	21	20	23 ± 3	1
20α- and 20β-dihydrocortisol	16	17	10	14 ± 3	2
Tetrahydrocortisol + 5α-tetrahydrocortisol	3	2	ND	2 ± 1	27
α- and β-cortol	2	4	8	5 ± 2	7
6β-hydroxycortisol	14	26	17	19 ± 4	1
Cortisone	23	18	31	24 ± 5	2
20α-and 20β-dihydrocortisone	8	6	6	7 ± 1	4
Tetrahydrocortisone	3	2	1	2 ± 1	35
α- and β-cortolone	3	5	7	5 ± 1	21

ND, not detectable.
[a]The data are presented as percentage of total corticosteroid metabolites excreted, as determined by gas chromatography–mass spectroscopy.
[b]The percentage of total metabolites excreted in human urine.[41]

Table 4. Relative amounts of 6β-hydroxycortisol (6β-F), cortisol (F), and cortisone (E) in urine[a] of male Bolivian squirrel monkeys

Animal no.	Osmolality of sample (mOsm/l)	6β-F (μg/dl)	F (μg/dl)	E (μg/dl)	6βF/F ratio	F/E ratio
2005	261	10	25	52	0.40	0.48
91011	572	101	119	136	0.85	0.88
3010	252	32	49	94	0.65	0.52
3050	127	17	31	64	0.56	0.48
3280	293	60	50	79	1.20	0.63
3612	438	44	45	81	0.98	0.56

[a]Urine was obtained from squirrel monkeys under ketamine–xylazine anesthesia.

physiologic parameters. Our results suggest that neither increased secretion of aldosterone nor a structural or functional change in the MR in squirrel monkeys contributes to protection. Rather, peripheral conversion of cortisol to inactive metabolites, excretion of free cortisol, and possibly other cellular events likely contribute to minimizing the effects of cortisol excess. These conclusions are based on the following findings: (1) circulating levels of aldosterone in squirrel monkeys fell within the normal human reference range; (2) we cloned and expressed the squirrel monkey MR in COS-7 cells and demonstrated that it is activated by cortisol and aldosterone in the same manner as is human MR; and (3) we found evidence of increased peripheral conversion of cortisol to cortisone and increased excretion of free corticosteroids as well as the cortisol metabolite 6β-hydroxycortisol.

Cortisol and cortisone are interconverted in the periphery by 2 enzymes, 11β-HSD1 and 11β-HSD2.[47,50] 11β-HSD1, which is most abundant in the liver, acts predominantly in vivo as a reductase, converting cortisone to cortisol. In contrast, the conversion of cortisol to cortisone is catalyzed by 11β-HSD2 that is found predominantly in kidney and intestine. The relative concentrations of cortisol and cortisone in serum are regarded as markers of the activities of the 2 enzymes. The ratio of cortisol to cortisone in the serum of humans ranges from 3 to 7,[23,26,42] but the ratio is higher in subjects with conditions of glucocorticoid excess, such as Cushing's syndrome.[14] This scenario is thought to result from exceeding the capacity of 11β-HSD2. However, the low ratio of cortisol to cortisone in squirrel monkey serum (approximately 1) suggests increased activity of 11β-HSD2, leading to enhanced peripheral conversion of cortisol to cortisone. A number of factors have been shown to influence 11β-HSD2 expression including corticosteroids.[46] It is also possible that low expression of 11β-HSD1 might contribute to the low ratio of cortisol to cortisone in squirrel monkeys, because Moore and colleagues showed low abundance of 11β-HSD1 mRNA in squirrel monkey tissues relative to levels in rat tissues.[27]

A comprehensive corticosteroid metabolite profile of urine collected from 3 animals confirmed that squirrel monkeys, like marmosets (another New World primate), excrete large amounts of free cortisol and cortisone.[38,39] Tetrahydrocortisol and tetrahydrocortisone are minor metabolites in squirrel monkey urine, unlike for humans. However, we found that 6β-hydroxycortisol makes up nearly 20% of the total corticosteroid metabolites excreted in squirrel monkeys. 6β-Hydroxycortisol is reported to be an important cortisol metabolite in some New World primates such as capuchin monkeys (*Cebus* spp.) but not marmosets (*Callithrix* spp.) or Guyanese squirrel monkeys.[3,38,39] 6β-Hydroxycortisol, only a minor metabolite in humans,[40] is formed from cortisol in a reaction catalyzed by 6β-hydroxylase. 6β-Hydroxylase is a member of the cytochrome P450 CYP3A family of drug- and hormone-metabolizing enzymes and is induced by macrolide antibiotics, such as rifampicin, and naturally occurring and synthetic steroids such as cortisol or dexamethasone.[32] As a consequence, the excretion of 6β-hydroxycortisol is increased in hypercortisolemic states.[49] It has been suggested that 6β-hydroxylase may serve as a 2nd enzyme involved in protecting MR from glucocorticoid occupancy.[28] Therefore, increased activity of several pathways of corticosteroid metabolism may contribute to the adaptive mechanisms used by squirrel monkeys to minimize the effects of excess cortisol.

It is likely that additional factors contribute to protection from high cortisol. Like squirrel monkeys, guinea pigs have high circulating cortisol levels and express apparently functional MR. Funder has suggested that MR in guinea pigs may always be occupied but not fully transcriptionally activated.[20] There may be several explanations that support this hypothesis. Progesterone, which is markedly elevated in squirrel monkeys,[8] may compete with cortisol for binding to unprotected MR.[29] In addition, MR may form dimers with glucocorticoid receptors, and the resulting heterodimers may have different transcriptional capacities compared with homodimers.[17,20] A different mechanism for protection is suggested from heterologous transfection studies such as the one presented here in Figure 3. In this and similar experiments performed by other investigators, MR activation, at least in cello,

requires significantly more glucocorticoid than aldosterone. This requirement suggests that cell-specific, likely nuclear, factors such as coactivators or corepressors participate in selective activation of MR by aldosterone over cortisol.[17,18]

Cytosolic factors have also been shown to affect responsiveness to steroid hormones. For example, Adams and colleagues discovered heat shock protein-70-like intracellular vitamin D-binding proteins in New World primate cells that facilitate intracellular targeting of vitamin D.[1] In addition, the steroid receptor cochaperone FKBP52 enhances glucocorticoid and androgen receptor signaling.[5,12,34] However, the related cochaperone FKBP51 is largely inhibitory to steroid receptor signaling.[13,34,51] It is not yet known whether any of these factors affect mineralocorticoid signaling and how they might contribute to adaptation to high cortisol in squirrel monkeys.

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