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Androgen Resistance in Squirrel Monkeys (*Saimiri* spp.)

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The goal of this study was to understand the basis for high androgen levels in squirrel monkeys (*Saimiri* spp.). Mass spectrometry was used to analyze serum testosterone, androstenedione, and dihydrotestosterone of male squirrel monkeys during the nonbreeding (n = 7) and breeding (n = 10) seasons. All hormone levels were elevated compared with those of humans, even during the nonbreeding season; the highest levels occurred during the breeding season. The ratio of testosterone to dihydrotestosterone in squirrel monkeys is high during the breeding season compared to man. Squirrel monkeys may have high testosterone to compensate for inefficient metabolism to dihydrotestosterone. We also investigated whether squirrel monkeys have high androgens to compensate for low-activity androgen receptors (AR). The response to dihydrotestosterone in squirrel monkey cells transfected with AR and AR-responsive reporter plasmids was 4-fold, compared with 28-fold in human cells. This result was not due to overexpression of cellular FKBP51, which causes glucocorticoid and progestin resistance in squirrel monkeys, because overexpression of FKBP51 had no effect on dihydrotestosterone-stimulated reporter activity in a human fibroblast cell line. To test whether the inherently low levels of FKBP52 in squirrel monkeys contribute to androgen insensitivity, squirrel monkey cells were transfected with an AR expression plasmid, an AR-responsive reporter plasmid, and a plasmid expressing FKBP52. Expression of FKBP52 decreased the EC_{50} or increased the maximal response to dihydrotestosterone. Therefore, the high androgen levels in squirrel monkeys likely compensate for their relatively low 5α-reductase activity during the breeding season and AR insensitivity resulting from low cellular levels of FKBP52.

Abbreviations: AR, androgen receptor; GR, glucocorticoid receptor; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MMTV, mouse mammary tumor virus; PR, progesterone receptor

Steroid hormones such as cortisol, aldosterone, estrogen, progesterone, and testosterone are lipophilic ligands for nuclear receptors that are part of a superfamily of transcription factors.[13,19] Upon biosynthesis, steroid hormones are released into the circulation to play essential roles in the regulation of diverse physiologic functions, including carbohydrate, protein and fat metabolism; electrolyte balance; and reproduction. Relative to Old World primates, squirrel monkeys (*Saimiri* spp.) and other New World primates have high levels of several steroid hormones including cortisol,[4] progesterone,[3] and testosterone.[14,30]

Initial studies suggested that elevated levels of cortisol arose to compensate for end-organ resistance to glucocorticoids.[4] Subsequent studies have shown that glucocorticoid resistance results at least in part from the expression of glucocorticoid receptors (GRs) that are inherently less capable of mounting a transcriptional response,[9,16,27] as well as from the overexpression in squirrel monkeys of the GR-Hsp90-associated cochaperone FKBP51 that reduces receptor binding affinity.[6,18] The immunophilin cochaperones FKBP51 and FKBP52 associate with many steroid receptors and can be important regulators of receptor function. A general model has been proposed whereby FKBP52 is stimulatory and FKBP51 is inhibitory to GR binding.[2,5,8] Because FKBP51 is higher and FKBP52 is lower in squirrel monkey cells and tissues compared with those from Old World species,[18,22] the relative levels of both immunophilins may contribute to glucocorticoid resistance in this species.

In addition, high circulating progesterone levels are thought to have arisen to compensate for progesterone resistance in squirrel monkeys.[3] The results of studies in which the effects of FKBP52 and FKBP51 on progesterone receptor (PR) function were directly analyzed[11,26,32] strongly suggest that, like the effect on GR function, altered cellular levels of these cochaperones contribute to impaired PR function in squirrel monkeys. These results suggest that multiple biochemical changes have led to glucocorticoid and progesterone resistance in squirrel monkeys. The reasons behind the high levels of testosterone in squirrel monkeys and other New World primates compared with Old World primates have drawn less attention and are investigated in the current study.

Several laboratories have reported that circulating levels of testosterone, its precursor androstenedione, and its 5α-reduced metabolite dihydrotestosterone undergo seasonal changes in adult, male squirrel monkeys, with the highest levels reached in the early part of the breeding season.[14,25,28] In contrast, marked variability in androgen levels that did not follow either diurnal or annual cycles also has been noted.[30] Nevertheless, all groups reported levels of androgenic hormones in New World primates that are

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higher than those in Old World primates and humans. Testosterone levels as high as 200 ng/ml in squirrel monkey serum have been measured, compared with levels in human males of approximately 4 ng/ml. Androstenedione levels of 150 ng/ml and dihydrotestosterone levels of 5 ng/ml (compared with 2 and 0.4 ng/ml in humans, respectively) were reported for male squirrel monkeys during the breeding season. Another study[25] reported a high ratio of testosterone:dihydrotestosterone (30 to 40) in squirrel monkeys compared with humans (ratio, 5 to 10). In that study, it was suggested that squirrel monkeys are 5α-reductase deficient and that high testosterone levels exist to compensate for reduced capacity to generate the more potent dihydrotestosterone. However, these previous studies used immunoassays to determine hormone levels, and recent analysis has stressed the limitations of this method for determination of testosterone.[21] This limitation may be exacerbated in squirrel monkeys and other New World primates that have high levels of other steroid hormones, potentially leading to assay interference. Accurate statements regarding annual cycles of androgenic hormones or 5α-reductase activity in squirrel monkeys require reanalysis of these hormones with the most rigorous methods available. The first goal of our study was to use a mass spectrometry approach to measure levels of testosterone, dihydrotestosterone, and androstenedione in adult, male squirrel monkeys during breeding and nonbreeding seasons.

Our second goal was to investigate whether androgen resistance at the tissue level in addition to 5α-reductase deficiency contributes to the high levels of androgens in squirrel monkeys. We hypothesized that differences in the cellular levels of the cochaperones FKBP51 and FKBP52 in squirrel monkeys result in alterations in androgen receptor function and compensatory increase in androgen secretion. Initial experiments suggested lower androgen receptor (AR) activity in squirrel monkey genital skin fibroblasts relative to human cells.[25] More recent studies using in vivo gene knockout and other experimental approaches have highlighted a role for these cochaperones in AR-mediated functions.[1,7,33] Therefore, we investigated how high levels of squirrel monkey FKBP51 and low levels of FKBP52 in squirrel monkey cells affect AR signaling. The results of our studies suggest that at least 2 factors lead to compensatory elevation of testosterone levels in squirrel monkeys during the breeding season: relatively low 5α-reductase activity and reduced responsiveness of the AR resulting from low cellular levels of FKBP52.

Materials and Methods

Serum androgen measurements. Subjects were adult, male Bolivian squirrel monkeys (*Saimiri boliviensis boliviensis*; age, 6 to 15 y) 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 commercial diet (New World Primate diet 5040, PMI Nutrition International, St Louis, MO) and seasonal produce. Peanuts and commercial treats (Prima-Treats, Bio-Serve, 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 samples were obtained by femoral venipuncture within 2 min of capture from 7 squirrel monkeys during the nonbreeding season and 10 squirrel monkeys during the breeding season; subjects were picked randomly from the colony. Serum was separated by centrifugation at 1200 × *g* for 20 min and stored at –80 °C. Testosterone, dihydrotestosterone, and androstenedione levels in serum were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) at the Mayo Medical Laboratories (http://mayoreferenceservices.org).

Cell cultures. Squirrel monkey 7603830 lung fibroblasts,[24] squirrel monkey SQMK-FP kidney epithelial cells (also called Pindak cells),[23] and human WI-38 VA-13 (WI-38) lung fibroblasts, purchased from American Type Culture Collection (Manassas, VA), were grown in DMEM supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 50 U/ml penicillin, and 0.05 mg/ml streptomycin. Human fibroblast T-REx-293-sm51 cells that overexpress squirrel monkey FKBP51 in a tetracycline-inducible manner were developed and maintained as previously described.[27] All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Transactivation assays. Androgen responsiveness was compared in squirrel monkey 7603830 and human WI-38 lung fibroblasts. Cells were plated in 6-well dishes at a density of 100,000 cells/well (WI-38) or 150,000 cells/well (7603830) to account for differences in growth rates. Cells were transiently transfected (Superfect, Qiagen, Valencia, CA) with the human AR expression vector pSG-hAR (0.5 µg/well; provided by Dr W E Zimmer, Texas A and M University, College Station, TX) and 1.5 µg/well mouse mammary tumor virus (MMTV) promoter–luciferase reporter vector (from Dr R M Evans, The Salk Institute, La Jolla, CA). Cells were grown in DMEM containing 10% charcoal–dextran-treated FBS (CD-FBS, Hyclone) for 24 h before treatment with 100 pM dihydrotestosterone (Steraloids, Wilton, NH). After 18 h, cells were washed, lysed, and assayed for luciferase activity by using a luciferase reporter assay kit (BD Biosciences Clontech, Palo Alto, CA).

The effect of overexpression of squirrel monkey FKBP51 on androgen, glucocorticoid, and progestin responsiveness was tested in T-REx-293-sm51 cells. Cells were plated in 6-well plates (Biocoat, BD Biosciences, Rockville, MD) at a density of 3×10^5 cells/well and were treated with 0.1 µg/ml tetracycline for 24 h to induce expression of squirrel monkey FKBP51. Cells were transiently transfected with pSG-hAR (0.5 µg/well) and either MMTV–luciferase or prostate-specific antigen enhancer–luciferase (PSE-luciferase, provided by Dr Michael Carey, University of California, Los Angeles, CA) vector (1.5 µg/well). Cells were grown in DMEM containing 10% CD-FBS for 24 h before treatment with dihydrotestosterone. In another set of cells, expression plasmids for GR (hGR-pcDNA1.1/AMP)[17] or PR (BKCMV-hPR-B vector, from Dr D P McDonnell, Duke University Medical Center, Durham, NC) were transfected (0.5 µg/well) with MMTV–luciferase vector (1.5 µg/well), and the cells were treated with dexamethasone (Sigma Chemical Company, St Louis, MO) or R5020 (NEN Life Science Products, Boston, MA), respectively. After 18 h, cells were collected and assayed for luciferase activity as described earlier. EC_{50} values (defined as the concentration of ligand that produces 50% of the maximal response) were obtained from the concentration–response curves.

The effect of transient overexpression of FKBP52 was tested in squirrel monkey 7603830 lung fibroblasts and squirrel monkey SQMK-FP kidney cells. 7603830 and SQMK-FP cells were plated in 6-well dishes at densities of 150,000 cells/well and 50,000 cells/well, respectively. Cells were transiently transfected with pSG-hAR plasmid (0.5 µg/well), PSE-luciferase reporter plasmid (1 µg/well), and either the FLAG-tagged rabbit FKBP52 expression vector (FKBP52-FLAG-pCMV5, provided by Dr Michael Chink-

Table 1. Levels of testosterone (T), dihydrotestosterone (DHT), and androstenedione (A) in male squirrel monkeys during the nonbreeding and breeding seasons

	Animal no.		T (ng/ml)	DHT (ng/ml)	T/DHT ratio	A (ng/ml)
Nonbreeding season						
	2560		15	2.7	5.6	139
	91011		17	2.0	8.5	122
	2212		6.9	1.5	4.6	44
	2439		8.4	1.0	8.4	46
	3688		4.6	0.6	7.7	16
	2569		2.5	0.7	3.6	15
	92186		18	2.1	8.6	64
		Mean ± SEM	10 ± 2	1.5 ± 0.3	6.7 ± 0.8	64 ± 19
Breeding season						
	2005	December	13	1.9	6.8	50
	2005	January	89	4.0	22	167
	2212		12	3.2	3.8	24
	2569		238	4.2	57	210
	3010		122	3.8	32	90
	92186		342	7.0	49	437
	2560		207	4.8	43	224
	92210		369	7.1	52	347
	3280		270	8.8	31	382
	2439		142	3.4	42	127
		Mean ± SEM	180 ± 40[a]	4.8 ± 0.7[a]	34 ± 6[a]	206 ± 45[a]
	Human[b]	Mean ± SEM	3.8 ± 0.1	0.33 ± 0.01	12	0.76 ± 0.02

Serum samples collected from 7 adult, male squirrel monkeys during the nonbreeding season (early September) and 10 squirrel monkeys during the breeding season (mid December – January) were assayed by LC-MS/MS.
[a]Significantly ($P < 0.05$) different from values from squirrel monkeys during the nonbreeding season or from humans.
[b]T, DHT, and A values for humans were determined by LC-MS/MS of serum from 160 subjects[10]

ers, University of South Alabama, Mobile, AL) or empty vector (0.5 µg/well). Cells were grown in DMEM containing 10% CD-FBS for 24 h before treatment with dihydrotestosterone. After 18 h, cells were collected and assayed for luciferase activity as described earlier.

Western blot analysis. Cell extracts were boiled in sample buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 7.5% gels. After transferring to nitrocellulose, blots were incubated in blocking buffer as described[27] for 1 h at room temperature. Incubation with the primary antibodies was carried out overnight at 4 °C. Antibodies to FKBP51 (hi51e) and FKBP52 (hi52b) were provided by Dr David Smith (Mayo Clinic Scottsdale, Scottsdale, AZ) and have been described elsewhere.[15] The AR antibody was from Upstate (Lake Placid, NY), and the anti-FLAG M2 antibody was from Stratagene (La Jolla, CA). The Hsp90 antibody was purchased from StressGen Biotechnologies (Victoria, BC Canada). All antibodies were used at a dilution of 1:1000, with the exception of hi52b (1:5000 dilution). After washing, blots were incubated with secondary antibodies and developed (Immune-Star Kit, Bio-Rad Laboratories, Hercules, CA).

Statistical analysis. Statistical analysis was performed by using GraphPad Prism version 4.0 software (San Diego, CA). Comparisons between 2 groups were performed by using an unpaired *t* test. Comparisons between more than 2 groups were performed by using 1-way ANOVA and the Newman–Keuls posthoc test. Values were considered statistically different when the *P* value was less than 0.05.

Results

Levels of testosterone, dihydrotestosterone, and androstenedione in squirrel monkeys. The first goal of this study was to reevaluate the levels of androgenic hormones in squirrel monkeys by using LC-MS/MS. Samples were obtained by femoral venipuncture from 7 adult, male squirrel monkeys during the nonbreeding season (early September) and 10 squirrel monkeys during the breeding season (mid December through January). The mean serum levels of testosterone in monkeys during the nonbreeding season (10 ± 2 ng/ml) were significantly ($P < 0.05$) lower than those reported by others using radioimmunoassay (52 ± 12 ng/ml),[28] but significantly ($P < 0.05$) higher than the levels usually seen in human subjects (3.8 ± 0.1 ng/ml)[10] (Table 1). During the breeding season, serum levels of testosterone were dramatically higher in most squirrel monkeys than the levels observed during the nonbreeding season.

The mean levels of the more potent androgen dihydrotestosterone were also significantly ($P < 0.05$) higher in squirrel monkeys than in humans (Table 1). Breeding season levels of dihydrotestosterone (4.8 ± 0.7 ng/ml) were significantly ($P < 0.05$) higher than nonbreeding season levels (1.5 ± 0.3 ng/ml), although the increase was not as large as that seen with testosterone. As a consequence, the testosterone:dihydrotestosterone ratio, which is approximately 7 in the nonbreeding season, rises to 34 in the breeding season.

The levels of circulating androstenedione in male squirrel monkeys during the nonbreeding season (64 ± 19 ng/ml) and breeding season (206 ± 45 ng/ml) determined by LC-MS/MS (Table 1) were not significantly different from those determined by using radioimmunoassay (91 ± 13 and 168 ± 15 ng/ml, respectively).[28] In both studies, a significant ($P < 0.05$) increase in circulating androstenedione occurred during the breeding season. Marked interanimal variability in androstenedione levels was present during both seasons, although within each animal levels of androstenedione correlated closely with the levels of the other androgens.

AR activity in squirrel monkey cells. The high level of androgenic hormones in squirrel monkeys, even during the nonbreeding season, compared with those in humans suggests a deficit in androgen receptor signaling. To test whether androgen responsiveness is inherently low in squirrel monkey cells, squirrel monkey 7603830 and human WI-38 lung fibroblasts were transiently transfected with human AR expression and MMTV-luciferase reporter plasmids, and the responses to dihydrotestosterone were compared. Treatment with 100 pM dihydrotestosterone induced luciferase activity in human WI-38 cells 28-fold above that of untreated cells, whereas induction in squirrel monkey 7603830 cells was only 4-fold (Figure 1 A). These results suggest that AR responsiveness is inherently low in squirrel monkey cells.

Androgen responsiveness is thought to be modified by Hsp90-associated cochaperones[1,7,33] that are expressed at different levels in squirrel monkey and human cells.[18,22] We demonstrated that FKBP51 is higher and FKBP52 is lower in squirrel monkey 7603830 cells than in human WI-38 cells (Figure 1 B). We then asked whether the different levels of these immunophilins might be responsible for the blunted AR response in squirrel monkey cells.

Effect of squirrel monkey FKBP51 on AR activity. Squirrel monkey FKBP51 is a potent inhibitor of GR and PR activity.[6,11] We therefore used T-REx-293-sm51 cells, which are derived from human 293 cells, to investigate whether overexpression of FKBP51 affects androgen responsiveness. T-REx-293-sm51 cells are an inducible, stable cell line that robustly expresses squirrel monkey FKBP51 when treated with tetracycline.[27] After plating, half of the dishes of T-REx-293-sm51 cells were treated with 0.1 µg/ml tetracycline for 24 h. Cells were transfected with the AR expression vector and either the MMTV–luciferase reporter (Figure 2 A) or the androgen-selective prostate-specific antigen enhancer–luciferase (PSE-luciferase) reporter plasmid (Figure 2 B). Cells were incubated for an additional 24 h before treatment with dihydrotestosterone and subsequently collected for determination of luciferase reporter activity. Western blots were performed to confirm the overexpression of FKBP51 (Figure 2 E). Expression of squirrel monkey FKBP51 had no significant effect on the response to dihydrotestosterone in T-REx-293-sm51 cells, regardless of which reporter vector was used. By using MMTV–luciferase as the reporter, the EC_{50} for dihydrotestosterone in control cells was 7.3 ± 3.8 pM, whereas the EC_{50} in cells expressing squirrel monkey FKBP51 was 12 ± 6.7 pM. Using the more selective PSE–luciferase as the reporter plasmid, the EC_{50} for dihydrotestosterone in control cells was 300 ± 15 pM, whereas the EC_{50} in cells expressing squirrel monkey FKBP51 was 350 ± 35 pM.

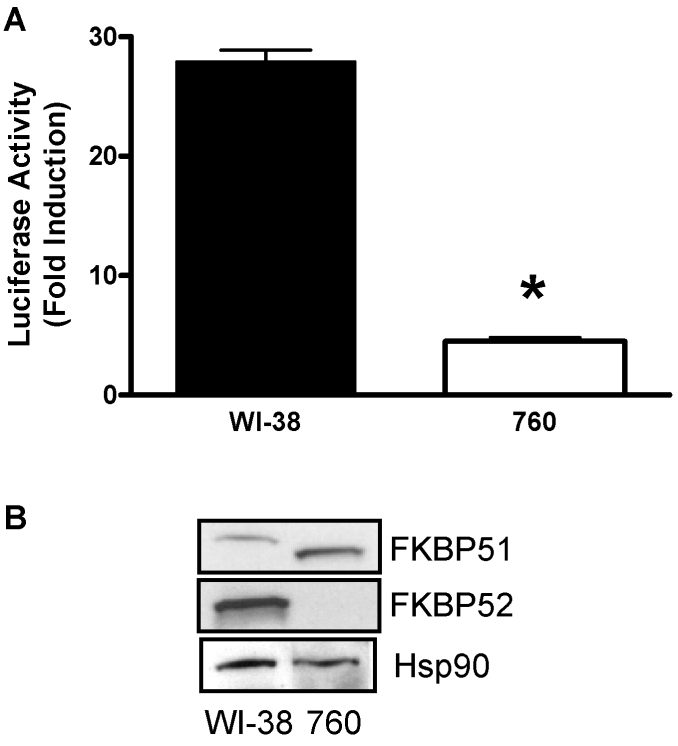

Figure 1. Reduced androgen responsiveness in squirrel monkey 7603830 lung fibroblasts. (A) Human WI-38 VA-13 (WI38) and squirrel monkey 7603830 (760) cells were transiently transfected with pSG-hAR expression and MMTV-luciferase reporter plasmids. After 24 h, cells were treated with 100 pM dihydrotestosterone, and luciferase assay was performed 18 h later. Data are expressed as fold-induction over values from untreated cells. The mean (bar, range) of 2 independent experiments is shown for each cell line. *, androgen responsiveness differed significantly ($P < 0.05$) between WI38 and 760 cells. (B) A representative Western blot shows the levels of FKBP51 and FKBP52 in WI38 and 760 cells. Hsp90 was used as an internal loading control.

In contrast to its effect on androgen responsiveness, expression of squirrel monkey FKBP51 in T-REx-293-sm51 cells reduced glucocorticoid and progestin responsiveness. Cells were transfected with GR or PR expression vectors and the MMTV-luciferase reporter plasmid and treated with either dexamethasone or the synthetic progestin R5020 before determination of reporter activity. The EC_{50} for dexamethasone was 10-fold higher in T-REx-293-sm51 cells expressing GR and squirrel monkey FKBP51 compared with control (2.1 ± 0.1 versus 0.21 ± 0.02 nM; Figure 2 C), whereas the EC_{50} for R5020 was 40-fold higher in cells expressing PR and squirrel monkey FKBP51 compared with control (40.8 ± 13.8 pM versus 1.0 ± 0.4 pM; Figure 2 D). Compared with results in control cells, the EC_{50} values for dexamethasone and R5020 were significantly ($P < 0.05$) different in cells in which the expression of squirrel monkey FKBP51 was induced. These results show that resistance to glucocorticoid and progestin is faithfully recapitulated in T-REx-293-sm51 cells expressing squirrel monkey FKBP51,

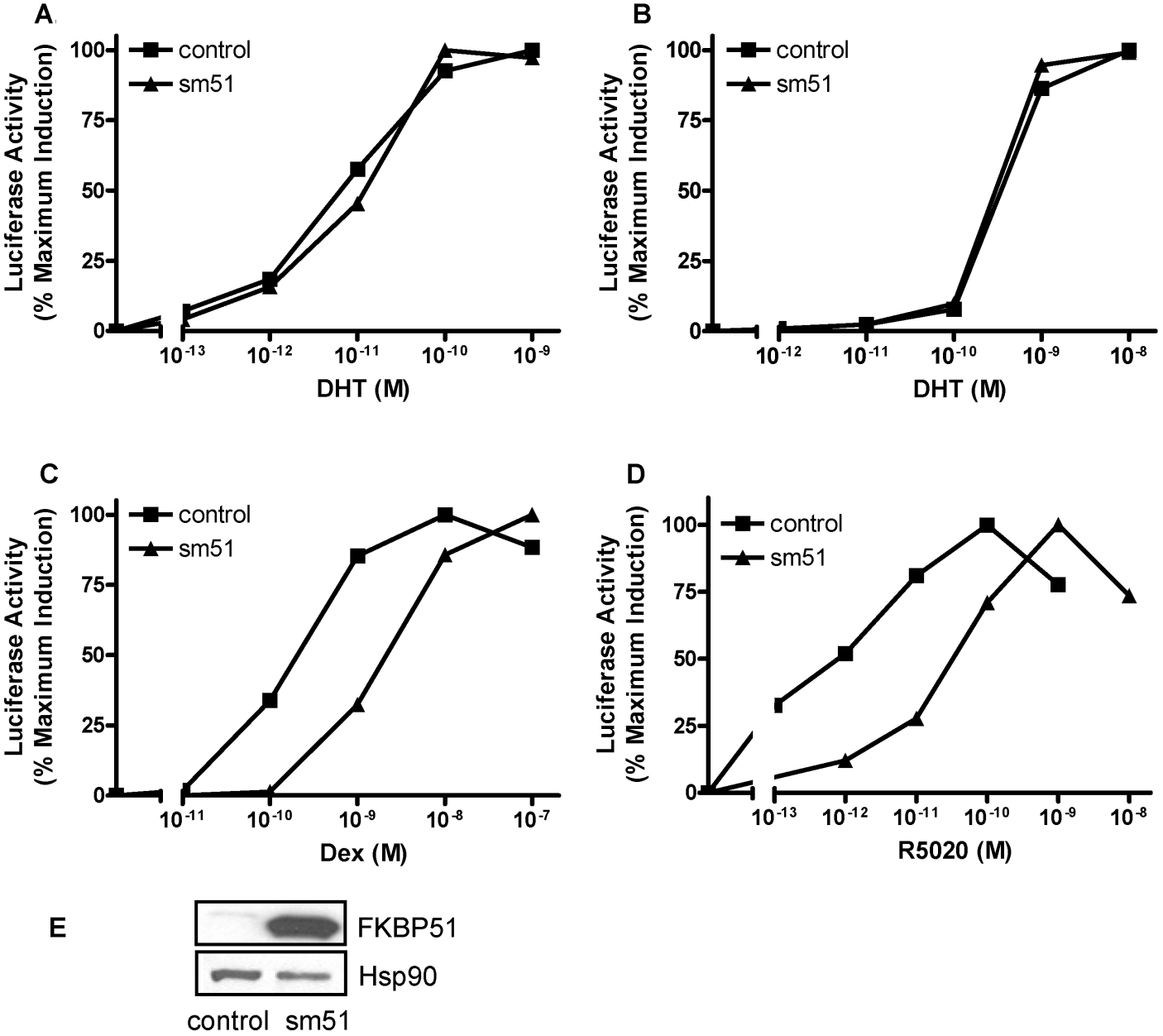

Figure 2. Effect of squirrel monkey FKBP51 on hormone-stimulated reporter gene activity in T-REx-293-sm51 cells. T-REx-293-sm51 cells were treated with vehicle (control) or 0.1 µg/ml tetracycline for 24 h to induce expression of squirrel monkey FKBP51 (sm51). (A, B) Cells were transiently transfected with pSG-AR expression and either (A) MMTV-luciferase or (B) PSE-luciferase reporter plasmids. (C, D) Cells were transfected with either (C) hGR-pcDNA1.1/AMP or (D) BKCMV-hPR-B and MMTV-luciferase reporter plasmids. After 24 h, cells were treated with either dihydrotestosterone (DHT; A, B), dexamethasone (Dex; C), or R5020 (D) and subsequently collected for assay of luciferase activity. Each point represents the mean from at least 3 independent experiments. (E) Expression of squirrel monkey FKBP51 in T-Rex-293-sm51 cells. Cell lysates from each transfected set were pooled, and Western blots were performed for FKBP51 and Hsp90 (used as an internal loading control).

as we and others have shown in other cell models.[6,11,31] The same level of expression of squirrel monkey FKBP51 had no effect on dihydrotestosterone-stimulated AR activity. Similar changes in androgen responsiveness were measured in CV1 cells with transient expression of squirrel monkey FKBP51 (data not shown). Likewise, expression of human FKBP51 in T-REx-293 or CV1 cells did not affect hormone-induced AR activity, suggesting that our findings were not due to a unique feature of squirrel monkey FKBP51.

Effect of FKBP52 on AR activity in squirrel monkey cells. Recent studies have shown that the structurally related immunophilin FKBP52 not only interacts with the AR but also enhances hormone-stimulated AR activity.[1,20,33] Because FKBP52 levels are lower in squirrel monkey cells and tissues compared with Old World species,[18,22,23] we asked whether low levels of FKBP52, rather than high levels of FKBP51, contribute to androgen insensitivity in squirrel monkey cells. To answer this question, we examined the effect of expression of FKBP52 on androgen respon-

siveness in 2 squirrel monkey cell lines transfected with AR and androgen-responsive reporter vectors. Squirrel monkey 7603830 lung fibroblasts and SQMK-FP kidney cells, like other squirrel monkey cell lines, express very low levels of FKBP52 relative to human cells.[22,23] Cells were transfected with AR expression vector, PSE–luciferase reporter plasmid, and either the plasmid FKBP52-FLAG-pCMV5 expressing rabbit FKBP52 or empty vector. After 24 h, cells were treated with dihydrotestosterone for 18 h before collection for assay of luciferase activity.

Expression of FKBP52 dramatically enhanced androgen responsiveness in both squirrel monkey cell lines, although the effect was qualitatively different. Expression of FKBP52 in 7603830 cells resulted in a significant ($P < 0.05$) 14-fold decrease in the EC_{50} for dihydrotestosterone-stimulated AR activity without a significant change in the maximal response to hormone (Figure 3 A). The EC_{50} was 290 ± 10 pM in control cells and 20.5 ± 3.5 pM in cells expressing rabbit FKBP52. Western blotting for AR and FLAG-tagged FKBP52 in 7603830 cells showed robust induction of FKBP52 in the presence of similar steady-state levels of AR (Figure 3 B). In SQMK-FP cells, FKBP52 overexpression had a different effect. In these cells, also transfected with AR expression and PSE–luciferase reporter gene vectors, FKBP52 overexpression resulted in a 3-fold induction in the response to maximal concentrations of dihydrotestosterone without a significant change in the EC_{50} values (Figure 4 A). Western blot analysis of cell lysates revealed that overexpression of FKBP52 in SQMK-FP cells resulted in a higher steady-state level of AR (Figure 4 B). Therefore, the higher response in SQMK-FP cells expressing FKBP52 likely results at least in part from increased expression of AR.

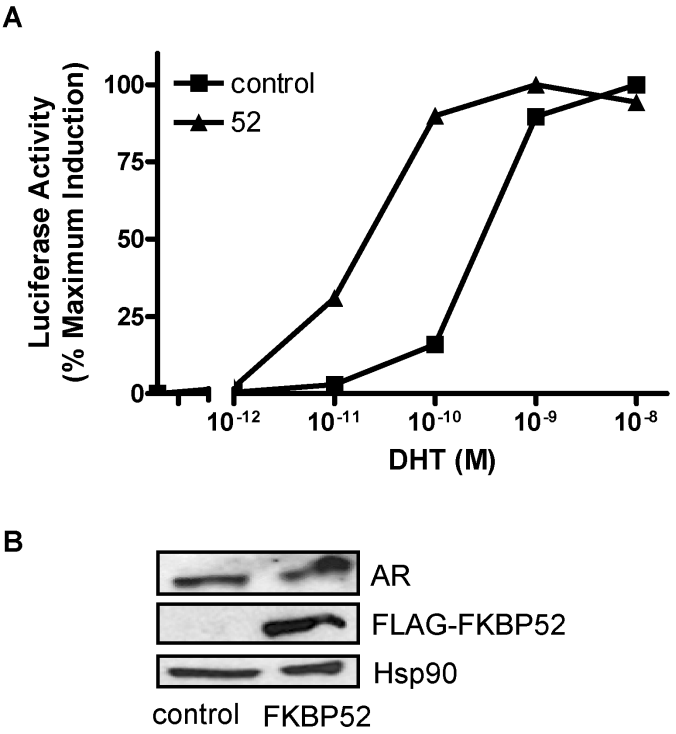

Figure 3. Effect of FKBP52 expression on androgen responsiveness in squirrel monkey 7603830 cells. (A) Cells were transfected with pSG-hAR, PSE-luciferase reporter plasmid, and either FKBP52-FLAG-pCMV5 (52) or empty vector. After 24 h, cells were treated with dihydrotestosterone (DHT) and subsequently collected for assay of luciferase activity. Each point represents the mean of 2 independent experiments. (B) Expression of AR, FLAG-tagged FKBP52 in 7603830 cells. Cell lysates from each transfected set were pooled, and Western blots were performed for AR, the FLAG epitope, and Hsp90 (used as an internal loading control).

Discussion

The levels of androgenic hormones reported in the literature for New World primates are elevated and vary considerably.[14,25,28,30] Furthermore, recent concerns have been voiced regarding the validity of some immunoassays for testosterone even in human samples.[21] The first goal of this study was to reevaluate the levels of these hormones in squirrel monkeys by using LC-MS/MS, which is a 'gold standard' method for quantification of sex steroid hormones. The use of LC-MS/MS provides a sensitive and reproducible assay method for determination of several steroid hormones in the same run. Here, we provide unequivocal measurement of circulating testosterone, dihydrotestosterone, and androstenedione levels in squirrel monkey serum during breeding and nonbreeding seasons. Using this method, we can confirm that, like the levels of cortisol and progesterone, the levels of androgenic hormones are elevated in squirrel monkeys, especially during the breeding season when greater variability in the levels was noted. Others have also reported inter- and intraanimal variability in circulating testosterone levels in male squirrel monkeys during the breeding season.[28,30] One of the most striking results was the testosterone:dihydrotestosterone ratio in squirrel monkeys, which is approximately 7 in the nonbreeding season but rises to 34 during the period of high testosterone secretion (that is, the breeding season).

In humans, the testosterone:dihydrotestosterone ratio is approximately 12. A ratio of approximately 35 in man is characteristic of a deficiency in the enzyme steroid 5α-reductase type 2.[12,29] Steroid 5α-reductase type 2 is the 5α-reductase isozyme expressed in external genital tissues, where it (1) plays an essential role in converting testosterone to dihydrotestosterone for local activity and (2) is a major contributor to circulating dihydrotestosterone.[29] Other researchers also have reported high testosterone:dihydrotestosterone ratios in squirrel monkey serum during the breeding season[25] and suggested that the high testosterone levels in these animals compensate in part for inefficient metabolism to dihydrotestosterone.

Our study also investigated whether androgen levels are high in squirrel monkeys to compensate for a deficit in AR signaling. We initially thought that elevated cellular FKBP51 might be responsible, given that it contributes to glucocorticoid and progestin resistance in squirrel monkeys. FKBP51 interacts with AR complexes,[7] but most studies (including ours) suggest that FKBP51 plays little role in regulating AR responsiveness. For example, FKBP51-deficient mice are phenotypically normal, exhibiting no evidence of changes in AR-mediated reproductive function.[33] In addition, overexpression of human FKBP51 had little effect on hormone-stimulated AR activity in yeast.[1,20] Modestly enhanced AR responsiveness is present in human prostate LNCaP cells overexpressing FKBP51,[7] suggesting that some cell contexts may support a small stimulatory effect of FKBP51 on AR responsiveness. Regardless, no studies support an inhibitory effect of FKBP51 on AR activity, leading to the conclusion that the low androgen responsiveness of squirrel monkey cells must occur by a mechanism other than overexpression of FKBP51.

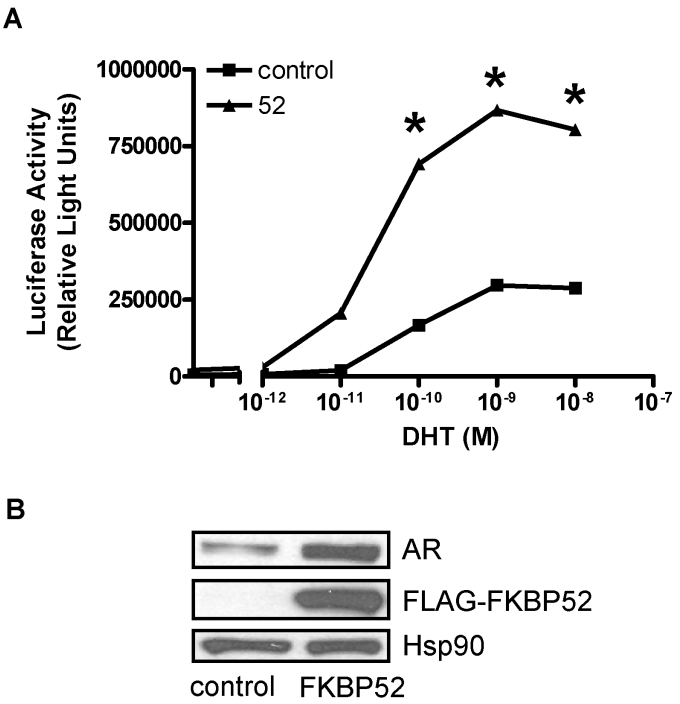

Figure 4. Effect of FKBP52 expression on androgen responsiveness in squirrel monkey SQMK-FP cells. (A) Cells were transfected with pSG-hAR, PSE-luciferase reporter plasmid, and either FKBP52-FLAG-pCMV5 (52) or empty vector. After 24 h, cells were treated with dihydrotestosterone (DHT) and subsequently collected for assay of luciferase activity. Each point represents the mean from 5 independent experiments. *, significantly ($P < 0.05$) different from activity in control cells. (B) Expression of AR, FLAG-tagged FKBP52 in SQMK-FP cells. Cell lysates from each transfected set were pooled, and Western blots were performed for AR, the FLAG epitope, and Hsp90 (used as an internal loading control).

Therefore, we asked whether low androgen responsiveness in squirrel monkey cells results at least in part from low expression of FKBP52. Studies in other systems also have shown that FKBP52 plays an important role in androgen responsiveness. Male mice lacking the gene encoding FKBP52 exhibited defects in a number of reproductive tissues, including hypospadias, nipples retained into adulthood, and dysgenic prostate and seminal vesicles.[1] These features are consistent with androgen insensitivity. Others recently confirmed these results by demonstrating that independently generated FKBP52 knockout mice likewise developed defects in select male reproductive organs.[33] How FKBP52 enhances AR responsiveness is not clear, but both Hsp90 binding and an intact peptidyl-prolyl isomerase domain are necessary for this activity.[1] The molecular effects of FKBP52 on AR are cell context-specific. The expression of FKBP52 in yeast[1] and in squirrel monkey 7603830 cells (Figure 3 A) resulted in a leftward shift in the androgen dose–response curve without a change in the maximal response, consistent with an effect of FKBP52 on AR binding affinity. However, other systems provide no evidence that FKBP52 affects AR hormone binding affinity or nuclear translocation,[1,33] suggesting that the shift in EC_{50} must occur through another mechanism. In HeLa cells,[1] mouse embryo fibroblasts,[33] and squirrel monkey SQMK-FP cells (Figure 4 A), FKBP52 expression increased reporter gene activity in response to maximal concentrations of hormone without a significant effect on the EC_{50} values. These results are consistent with an effect of FKBP52 on transcriptional activation. Studies in HeLa and SQMK-FP cells suggested that FKBP52 increases the steady-state levels of the AR, leading to enhanced hormone-stimulated AR activity. Yet unknown are how FKBP52 affects the stability of AR and whether the low concentration of FKBP52 in squirrel monkey tissues is naturally associated with decreased levels of AR.

Taken together, these results suggest that at least 2 factors contribute to the increased levels of androgenic hormones in New World squirrel monkeys. We propose that (1) low expression of FKBP52 results in impaired AR responsiveness and (2) lower 5α-reductase activity during the breeding season in squirrel monkeys relative to other species leads to a compensatory increase in androgen production.

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