Steroid Resistance in the Squirrel Monkey: An Old Subject Revisited

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Introduction

It has been more than 30 yr since Brown et al. (1968) reported at the 52nd Annual FASEB Meeting in Atlantic City that squirrel monkeys have high circulating levels of the adrenal steroid cortisol compared with humans. This work was subsequently published in full (Brown et al. 1970) and confirmed by other laboratories (for example, Chrousos et al. 1982; Coe et al. 1978; Klosterman et al. 1986; Manogue et al. 1975). In the meantime, it was recognized that other steroid hormones, including progesterone, 17β-estradiol, and testosterone, are also elevated in squirrel monkeys (Mendoza et al. 1978; Wilson et al. 1978; Wolf et al. 1977). Since these discoveries, a number of reports have contributed to our understanding of these phenomena, although not until recently have we begun to appreciate the molecular changes that led to these elevated hormone levels.

The goal of this article is not to review our current knowledge of secretion and metabolism of each of these steroids in the squirrel monkey. Such review has been expertly achieved in chapters published in a volume of Advances in Experimental Medicine and Biology dedicated to the late Dr. Mortimer Lipsett (Chrousos et al. 1986a, b; Siiteri 1986). Rather, this article details recent findings that have provided insight into the molecular events that led to hypercortisolemia, briefly describing their relevance to the secretion of other steroid hormones in squirrel monkeys.

The issue of elevated steroid hormone levels is important to laboratory animal science for several reasons. First, it should be appreciated that high circulating levels of some steroid hormones are a normal finding in captive squirrel monkeys. Second, it is generally agreed that this phenomenon has occurred as a normal physiological response to a state of hormone insensitivity or resistance in responsive tissues, including tissues such as the hypothalamus and pituitary gland, which mediate the feedback response. As a consequence, hormone levels are high to compensate for end-organ resistance, and squirrel monkeys enjoy a relatively normal pituitary-adrenal physiology albeit at a higher hormonal set-point. Thus, cortisol secretion in squirrel monkeys is appropriately stimulated by chair restraint (Brown et al. 1970) and social and environmental perturbations (Coe et al. 1982). At the same time, squirrel monkeys are relatively insensitive to the effects of administered steroid. For example, squirrel monkeys require almost 50-fold more dexamethasone to achieve a 50% suppression of plasma cortisol levels compared with cynomolgus monkeys and standardized to body weight (Chrousos et al. 1982). This requirement must be taken into account when administering steroid hormones to squirrel monkeys.

A similar note of caution should be applied to steroid responsiveness in other, but not all, neotropical primates. Monkeys in the Callitrichidae family, which includes marmosets and tamarins, consistently exhibit very high cortisol levels that suggest glucocorticoid resistance (Chrousos et al. 1982; Klosterman et al. 1986). However, within the Cebidae family, which includes the squirrel monkey, several other monkeys (such as owl monkey, titi monkey, and capuchin) have lower cortisol levels, suggesting only a modest level of glucocorticoid resistance. The reason for genus-specific differences in glucocorticoid resistance among neotropical primates is unknown.

Free Level of Cortisol in Squirrel Monkey Serum

Cortisol exists in the circulation as free hormone and hormone bound to serum proteins, predominantly corticosteroid-binding globulin and albumin. It is thought that only free hormone can diffuse into target cells and activate intracellular receptors. As discussed above, the total level of cortisol (that is, the sum of free and protein bound cortisol) in squirrel monkeys is more than 10 times that in humans. Even more dramatic is the difference in the amounts of circulating free cortisol. Whereas only 4 to 6% of total cortisol in human serum is unbound, more than one third of cortisol in squirrel monkey serum is free, translating into a free concentration of cortisol that is approximately 100 times greater than that found in human serum (Klosterman et al. 1986). As a consequence, large amounts of unmetabolized cortisol are excreted in the urine of squirrel monkeys (Setchell et al. 1977).

Cortisol is maintained at a high level in squirrel monkeys by several physiological changes. First, high levels of adrenocorticotropic drive increased synthesis and secretion of cortisol from the adrenal gland (Cassorla et al. 1982; Chrousos et al. 1984). This action occurs in squirrel monkeys with the backdrop of low expression of corticosteroid-binding globulin, which has a reduced affinity for cortisol.
Not only is there a low rate of metabolic clearance of cortisol (Cassorla et al. 1982), but also peripheral 11β-hydroxysteroid dehydrogenase in squirrel monkeys appears to favor conversion of inactive cortisone to cortisol (Moore et al. 1993). Thus, squirrel monkeys possess multiple mechanisms to maximize the levels of free cortisol to compensate for end-organ resistance.

Researchers in several laboratories have investigated the biochemical bases of lower glucocorticoid responsiveness in squirrel monkeys. Chrousos et al. (1982) found that glucocorticoid receptors (GRs1) in circulating mononuclear leukocytes exhibit a 20-fold lower binding affinity than GR in either human or rhesus monkey leukocytes, although the number of receptors did not differ among the cells. Both receptor density and binding affinity levels in fibroblasts cultured from squirrel monkeys were found to be lower than in human fibroblasts (Chrousos et al. 1982; Siiteri et al. 1982). It should be noted that these studies refer to changes in the affinity of the type II glucocorticoid receptor, not the type I receptor that mediates the physiological response to aldosterone (Arriza et al. 1987; Krozowski and Funder 1983). In fact, the level of plasma aldosterone is less than two-fold higher in squirrel monkeys compared with cynomolgus monkeys (Chrousos et al. 1984), suggesting that physiological responsiveness to aldosterone is not markedly different in squirrel monkeys. Colleagues and I began to investigate glucocorticoid resistance in squirrel monkeys as part of an ongoing interest in squirrel monkey endocrinology, and the investigation was predominantly carried out by Philip Reynolds (1998).

**Squirrel Monkey Cell Lines**

Experiments conducted in the laboratories of the Departments of Pharmacology and Comparative Medicine at the University of South Alabama have developed continuous squirrel monkey cell lines as an alternative to fresh tissues from this relatively scarce and expensive research model. One cell line exhibits the species-specific phenotype of glucocorticoid resistance and provides an unlimited supply of DNA, mRNA, and protein from the squirrel monkey. The lung fibroblast DPSO 114/74 cell line, which was previously the only squirrel monkey cell line available for general distribution from American Type Culture Collection, has a limited life span (<25 passages). Although Drs. Herberling and Kalter (Virus Reference Laboratory, San Antonio, Texas) and Frank Pindak (University of South Alabama) had generously provided a number of lung and kidney fibroblast lines, the lines have yet to be fully characterized.

Because B-lymphoblasts transformed with Epstein-Barr virus had been commonly used for studies of GR function in human cells (for example, Hurley et al. 1991), colleagues and I chose to develop a B-lymphoblast cell line from the squirrel monkey. Because transformation of B-lymphocytes from neotropical primates (including squirrel monkeys) with Epstein-Barr virus had not been uniformly successful (for example, Miller et al. 1972), we decided to modify existing techniques. The details of the successful transformation of squirrel monkey and (in a separate experiment) owl monkey lymphocytes have been published (Reynolds et al. 1998; Scammell et al. 1997). These cell lines are available from American Type Culture Collection (CRL 2311 and CRL 2312, respectively). We showed that the squirrel monkey lymphoblast line exhibits cellular morphology characteristic of B-cell lineage (Figure 1) and the species-specific phenotype of glucocorticoid resistance (Reynolds et al. 1998).

Using whole cell binding analysis, we further established that the insensitivity of these squirrel monkey cells to glucocorticoids results at least in part from expression of a low-affinity GR (Reynolds et al. 1997).

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1 Abbreviations used in this article: GR, glucocorticoid receptor; hsp, heat shock protein.

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**Figure 1** Development of an immortalized squirrel monkey B-lymphoblast cell line. Analysis of these cells by scanning electron microscopy revealed that they have numerous microvillous projections indicative of B-cell lineage. Magnification x6000. Reproduced with permission from Reynolds PD, Roveda KP, Tucker JA, Moore CM, Valentine DL, Scammell JG. 1998. Glucocorticoid-resistant B-lymphoblast cell line derived from the Bolivian squirrel monkey (Saimiri boliviensis boliviensis). Lab Anim Sci 48:364-370.
**Expression of the Squirrel Monkey Glucocorticoid Receptor**

The cloning and sequencing of the squirrel monkey glucocorticoid receptor was a first step in understanding the molecular basis of the low binding affinity of the squirrel monkey GR. A number of reports have shown that low binding affinity of the GR results from point mutations in the receptor gene, leading most often to single amino acid changes in the ligand binding domain (reviewed by Brönnegård and Carlstedt-Duke 1995). Indeed, it was originally postulated that glucocorticoid resistance in another neotropical primate, the cotton-top tamarin, resulted from amino acid substitutions or deletions (Brandon et al. 1991; Tomita et al. 1986). Colleagues and I used reverse transcription-polymerase chain reaction to clone the squirrel monkey GR and compare the sequence with that of the human GR. This work has been published in detail (Reynolds et al. 1997).

Our findings revealed that the squirrel monkey receptor is 97% identical in nucleotide and amino acid sequence to the human receptor’s sequence. The ligand binding domain (amino acids 528 to 777) of the squirrel monkey GR contains four amino acid differences: Ser551 to Thr, Ser616 to Ala, Ala618 to Ser, and Ile671 to Leu. All of these amino acid changes are conservative (i.e., the amino acids substituted have similar chemical characteristics), but even conservative mutations (such as Val729 to Ile) can significantly affect GR binding affinity (Malchoff et al. 1993). To determine whether the amino acid substitutions in the ligand binding domain are responsible for the decreased affinity of the squirrel monkey GR, colleagues and I examined the binding of human and squirrel monkey receptors expressed in vitro in a commercially available reticulocyte lysate system. After translation, we subjected the reaction to an ATP-regenerating system that fosters the formation of the GR heterocomplex. We found that when expressed in this system, squirrel monkey and human GRs had similar high-affinity binding for dexamethasone (apparent $K_d$ of 5.9 and 4.3 nM, respectively), whereas a mutant squirrel monkey GR (Phe774 to Leu) known to have low affinity was faithfully expressed and its low affinity reproduced (Reynolds et al. 1997). Thus, the amino acid substitutions within the ligand binding domain of the squirrel monkey GR cannot account for its low binding affinity. Rather, our results suggested that the cytosolic milieu of squirrel monkey cells (perhaps components of the GR heterocomplex) affects the binding affinity of the squirrel monkey GR.

**Analysis of GR-associated Proteins**

To demonstrate that squirrel monkey cells express soluble inhibitory factors, GR binding was determined in cytosol from mouse L929 cells, a rich source of high-affinity GR, mixed 1:1 with cytosol from either squirrel monkey lymphoblasts or COS-7 cells. Adding cytosol from squirrel monkey cells to L929 cell cytosol resulted in an 11-fold decrease in binding affinity (Figure 2). However, neither the nature of this factor nor the manner in which it affects GR binding was identified. Because the binding activity of GR is highly dependent on the ordered assembly of a mature receptor heterocomplex (Figure 3, top panel), it was speculated that the low binding activity of squirrel monkey GR might result from a deficiency in or a mutation of one of the components of the heterocomplex.

**Figure 2** The addition of cytosol from squirrel monkey cells to cytosol from L929 cells lowers glucocorticoid receptor (GR) binding affinity. (A) Saturation curves of [3H]dexamethasone binding in L929 cytosol mixed 1:1 with either COS-7 cell cytosol (L+COS7) or squirrel monkey lymphocytes (SML) cell cytosol (L+SML). (B) Scatchard plots of GR binding. L929 cells were used as a source of high-affinity GR. COS-7 cells express very low levels of GR and were used as a control. Reproduced with permission from Reynolds PD, Ruan Y, Smith DF, Scammell JG. 1999. Glucocorticoid resistance in the squirrel monkey is associated with overexpression of the immunophilin FKBP51. J Clin Endocrinol Metab 84:663-669.
Figure 3 Western blot analysis of glucocorticoid receptor (GR)-associated proteins in cytosols from human lymphocytes (HL) and squirrel monkey lymphocytes (SML) demonstrates a higher level of FKBP51 and a lower level of FKBP52 in SML. (Top) Schematic representation of GR heterocomplex assembly (adapted from Pratt and Toft 1997). (Bottom) The relative levels of each component of the GR heterocomplex. The identity and size of each protein are labeled above and to the left of each composite, respectively. Cyclophilin 40 (CyP40), protein phosphatase 5 (PP5), FKBP52, and FKBP51 can occupy the immunophilin (Imph) site on hsp90. Reproduced with permission from Reynolds PD, Ruan Y, Smith DF, Scammell JG. 1999. Glucocorticoid resistance in the squirrel monkey is associated with overexpression of the immunophilin FKBP51. J Clin Endocrinol Metab 84:663-669.

As a first step in identifying inhibitory factors in squirrel monkey cells, my colleagues and I compared the levels of components of the GR heterocomplex in squirrel monkey and human lymphoblasts. Of the proteins for which we probed, the most dramatic differences between squirrel monkey and human cells were found in the levels of the immunophilins FKBP51 and FKBP52 (enzymes that catalyze the trans to the cis configuration needed to promote protein folding). Compared with levels in human cells, FKBP51 was more than 10-fold higher in squirrel monkey cells, whereas FKBP52 was approximately one half that in human cells (Figure 3, lower panel). We also discovered that the inhibitory effect of cytosol from squirrel monkey cells on GR binding was reproduced using cytosol from COS-1 cells transiently transfected with an expression plasmid encoding squirrel monkey FKBP51 (Figure 4), supporting the conclusion that the inhibitory factor in squirrel monkey cells is FKBP51. Details of these experiments have been published (Reynolds et al. 1999).

How does higher expression of FKBP51 cause low binding affinity? FKBP51 and FKBP52 are FK506 (tacrolimus, an immunosuppressive drug used in organ transplantation)-binding immunophilins that, with PP5 and Hop, possess tetratricopeptide repeat motifs and compete for a common binding site on heat shock protein (hsp)^1^ 90. The makeup of hsp90 complexes is determined by the levels and affinities of these proteins for the hsp90 binding site (reviewed by Pratt and Toft 1997). High-affinity GR heterocomplexes in L929 cells contain FKBP52 or PP5, but little FKBP51 (Silverstein et al. 1997). However, L929 cells, like human lymphocytes or COS cells, express very little FKBP51. When FKBP51 is present during receptor assembly, at a level comparable with other immunophilins, it is preferentially incorporated into GR complexes (Barent et al. 1998). We noted in our cytosol mixing studies that the decrease in GR binding was associated with incorporation of squirrel monkey FKBP51 into GR heterocomplexes (Figure 5). How the presence of FKBP51 in the complex leads to low-affinity binding is not yet known,
but possible mechanisms have been discussed in detail elsewhere (Reynolds et al. 1999). In addition, in the course of our experiments, we were surprised to learn that squirrel monkey FKBP51 was several fold more potent than human FKBP51 in inhibiting GR binding. Although the amino acid sequence of squirrel monkey FKBP51 is unique in only 28 positions compared with human FKBP51, structural differences may permit squirrel monkey FKBP51 to preferentially incorporate into the GR heterocomplex or, once incorporated, to have a greater influence on GR binding than the human protein. Thus, FKBP51 overexpression and perhaps specific FKBP51 sequence differences appear to be the major causes of glucocorticoid resistance in the squirrel monkey.

**Other Effects of Elevated KFBP51**

In addition to elevated cortisol, squirrel monkeys exhibit uncommonly high levels of other steroid hormones, and monocytes derived from squirrel monkeys secrete very low levels of interleukin-1β and high levels of transforming growth factor β compared with humans (Hinze-Selch et al. 1997). Because hsp90 is required to maintain a functional hormone binding domain in the progesterone receptor as well as the GR (Pratt and Toft 1997), changes in immunophilin expression or activity may also be the cause of progesterone resistance in the squirrel monkey.

Less is known about the composition of androgen and estrogen receptor complexes, and their hormone binding conformations may be less strictly dependent on chaperone interactions (Fang et al. 1996; Nair et al. 1996). Proof that overexpression of FKBP51 mediates resistance to progesterone, androgen, or estrogen in the squirrel monkey awaits additional investigation. In any case, the effects of squirrel monkey FKBP51 are likely dependent on its association with hsp90 (Figure 3, top panel). Hsp90 is known to complex with a number of proteins, but it is thought that FKBP51- or FKBP52-associated hsp90 complexes only with steroid hormones (Pratt 1998; Toft 1998). Thus, it is unlikely that the activity of any of the many other targets of hsp90 (such as Raf or pp60v-src) is affected by overexpression of FKBP51 in squirrel monkeys. However, the cellular effects of elevation in uncomplexed FKBP51 are completely unknown. At the time of this writing, hsp90 is the only known target of FKBP51. Although Chambraud et al. (1996) identified a protein termed FAP46, which forms a complex with FKBP52, it is not known whether it associates with FKBP51 or affects function when bound to either immunophilin.

**Changes in Immunophilin Expression**

Also unknown is why these reciprocal changes in FKBP51 and FKBP52 expression in squirrel monkeys occur. Presumably they arose 25 to 50 million yr ago during the course of independent evolution of these and certain other primates on the South American subcontinent in response to different...
dietary and environmental stresses. They may have occurred to alter the sensitivity to certain steroid hormones or as a consequence of an insult independent of the endocrine system. The expression of FKBP51 messenger RNA is increased by glucocorticoids (Baugham et al. 1997; Reynolds et al. 1998), but otherwise, nothing is known about the factors that regulate the expression of these proteins.

Regulatory regions of the FKBP51 gene are beginning to be cloned and characterized. Such gains should provide insight into the nature of these unknown physiological or biochemical stressors. Regardless of the causes of the changes in immunophilin expression and structure, this event in the squirrel monkey has provided an intriguing experiment of nature that has lent itself to the discovery of a previously unrecognized mechanism for regulating steroid sensitivity.

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References


