**UCSF**
**UC San Francisco Electronic Theses and Dissertations**

**Title**
Modulation of glucocorticoid action by 11B-hydrosysteroid dehydrogenase (11B-oxoreductase)

**Permalink**
https://escholarship.org/uc/item/3bd0w56f

**Author**
Hammami, Muhammad Maher

**Publication Date**
1990

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

Modulation of glucocorticoid action by
11β-Hydroxysteroid dehydrogenase (11β-oxoreductase)

by

Muhammad Maher Hammami, M.D.

**DISSERTATION**

**Submitted in partial satisfaction of the requirements for the degree of**

**DOCTOR OF PHILOSOPHY**

**in**

Endocrinology

**in the**

**GRADUATE DIVISION**

**of the**

**UNIVERSITY OF CALIFORNIA**

**San Francisco**

# DEDICATION

**To Muhammad(570-632),**

**the illiterate,who taught**

**that seeking knowledge per se is rewardable, and**

**how to evaluate knowledge,**

**its truthfullness and its usefullness.**

## ACKNOWLEDGMENTS

To Dr. Pentti K. Siiteri. Being too short to address your knowledge, wisdom and contributions, I say thank you Finn for your trust, support and encourgement without which this work would have not been accomplished.

I thank Dr. Richard Weiner and Dr. Mary Dallman for their valuable time and timely suggestions as advisors and teachers and for serving on my oral committee and thesis committee.

I acknowledge the silent and nondemanding help and advice from Dr. James Murai.

I thank Adrienne Bronstein and Jimmy Choy for their help, good humor and friendship and Louise Masewicz for kindly typing this thesis.

MODULATION OF GLUCOCORTICOID ACTION BY 11ß-HYDROXYSTEROID DEHYDROGENASE (11ß-OXIDOREDUCTASE)

Muhammad M. Hammami, M.D.

Abstract

To characterize the activity of 11ß-hydroxysteroid dehydrogenase complex, its regualtion and its modulation of glucocorticoid (GC) action, 11ß-dehydrogenase and 11-oxo-reductase activities were studied in GC-sensitive (human) and GC-resistant (squirrel monkey, Sm) skin fibroblasts by incubating intact cells with [$^3$H] cortisol or [$^3$H] cortisone and determining their interconversion by thin layer chromatography. In human cells 11-oxo-reductase activity was 5-10 fold higher than 11ß-dehydrogenase activity due to a correspondingly higher Vmax. Both activities were increased by glucocorticoids and decreased by insulin, cAMP or phorbol esters. These hormones/second messengers were ineffective in regulating 11ß-HSD activity in Sm cells ; and 11ß-HSD activity was several hundred fold higher than that in human or rhesus monkey cells. Further, in Sm cells, the ratio of 11ß-dehydrogenase activity to 11-oxo-reductase activity was one. Changing the temperature , or the glucose content ,of the medium altered the 11ß-dehydrogenase to 11-oxo-reductase activity ratio in Sm cells suggesting its dependence on the ratio of the cofactors ,NADP and NADPH. The effectiveness of cortisone in inducing aromatase activity in human cell lines paralleled endogenous 11ß-HSD activity. 11ß-HSD activity in Sm cells was inhibited by a wide variety of steroids including progesterone and testosterone. The same steroids inhibited binding of [$^3$H] cortisone to these cells with similar potency suggesting that the binding is to 11ß-HSD and that the inhibition is competitive. This was confirmed by affinity labeling of 11ß-HSD using [$^3$H] dexamethasone mesylate. Furthermore, cortisone,

androstenedione and progesterone had similar potency in inhibiting [$^3$H] progesterone or [$^3$H] androstenedione binding to, and metabolism by, Sm cells. We conclude that: 1) 11ß-HSD is a single protein that catalyzes the interconversion of cortisol and cortisone and is under multifactorial regulation, 2) 11ß-HSD may increase or decrease cortisol availability to glucocorticoid receptors depending on the redox state of cells, 3) 11ß-HSD is constitutively overexpressed in Sm cells, 4) high 11ß-HSD activity and increased 11ß-dehydrogenase to 11-oxo-reductase activity may play a primary role in glucocorticoid and other steroid resistance in squirrel monkeys, 5) GC activity may be modulated by other steroid hormones via competition for 11ß-HSD, and 6) Sm cells and affinity labeling of 11ß-HSD with cortisone mesylate provides an efficient way to characterize further this enzyme at a molecular level.

Table of Contents

1. Introduction (1).

2. Literature review:

a- 11ß-Hydroxysteroid dehydrogenase (3)

b- Cortisol resistance and hypersensetivity.(8)

c- Cortisone- the neglected precursor.(19)

2. Regulation of 11ß-Hydroxysteroid dehydrogenase activity in human skin fibroblasts: Enzymatic modulation of glucocorticoid action (22).

a - Abstract (24)

b - Introduction (25)

c - Materials and Methods (27)

d - Results (30)

e - Discussion (34)

f - References (39)

g - Figure legends (44)

h - Tables (48)

i - Figures (51a)

3. 11ß-Hydroxysteroid dehydrogenase and glucocorticoid resistance in new world primates (52).

a - Abstract (53)

b - Introduction (54)

c - Materials and Methods (55)

d - Results (58)

e - Discussion (62)

f - References (67)

g - Tables (72)

h - Figure legends (75)

i - Figures (76a)

4. Specificity, steroid binding and affinity labeling of 11ß-hydroxysteroid dehydrogenase: Role in general steroid resistance (77)

a - Abstract (79)

b - Introduction (80)

c - Results (81)

d - Discussion (85)

e - Materials (89)

f - Methods (90)

g - Tables (94)

h - Figure legends (99)

i - References (101)

k - Figures (104a)

5 Summary and Conclusions (105)

6. References.

List of Tables

- Regulation of 11ß-hydroxysteroid dehydrogenase activity in human skin fibroblasts: enzymatic modulation of glucocorticoid action.

  Table 1 Cortisol induction of fibroblasts 11-oxo-reductase activity in the presence or absence of glucocorticoid receptor antagonists(48).

  Table 2 Modulation of 11ß-HSD activity by serum, glucocorticoids and insulin (49).

  Table 3 Kinetic studies of basal and induced 11-oxo-reductase and 11ß-dehydrogenase activities (51).

- 11ß-Hydroxysteroid dehydrogenase and glucocorticoid resistance in new world primates.

  Table 1 11ß-HSD activity in Sm, Rm and Hu cell lines (72).

  Table 2 Kinetic studies of 11ß-HSD in Sm skin fibroblasts incubated under different condition. (74).

- Specificity, steroid binding and affinity labeling of 11ß-hydroxysteroid dehydrogenase: Role in general steroid resistance.

  Table 1 Inhibition of 11-oxo-reduction of [$^3$H] cortisone and 11ß-dehydrogenase of [$^3$H] cortisol by other steroids (94).

  Table 2 [$^3$H] cortisone binding to Sm cells (96).

  Table 3 Inhibition of [$^3$H] cortisone binding to Sm cells (97).

List of Figures


- Regulation of 11ß-Hydroxysteroid dehydrogenase activity in human skin fibroblasts: Enzymatic modulation of glucocorticoid action

Fig. 1 Basal 11-oxo-reductase and 11ß-dehydrogenase activities in human genital skin fibroblasts (51a)

Fig. 2 Specificity of glucocorticoid induction of 11ß-HSD (51b)

Fig. 3 Dexamethasone induction of 11ß-HSD ( 51c)

Fig. 4 Inhibitory effect of serum on 11-oxo-reductase activity (51d)

Fig. 5 Insulin inhibition of 11ß-HSD activity in cells grown in serum free medium (51e)

Fig. 6 Aromatase induction by cortisol or cortisone (51f)

- 11ß-hydroxysteroid dehydrogenase and glucocorticoid resistance in new world primates

Fig. 1 11ß-HSD activity, saturation plot (76a)

Fig. 2 11ß-HSD activity, time course (76b)

Fig. 3 11ß-HSD activity in glucose free medium (76c)

Fig. 4 11ß-HSD activity, time course at 4° (76d)

Fig. 5 11ß-HSD regulation (76e)

- Specificity, steroid binding and affinity labeling of 11ß-hydroxy steroid dehydrogenase: Role in general steroid resistance

Fig. 1 [$^3$H] cortisone binding to Sm cells at 4° (104a)

Fig. 2 Affinity labeling of 11ß-HSD by [$^3$H] dexamethasone mesylate (104b)

Fig. 3 Inhibition of reduction of androstenedione to testosterone and progesterone to 20 α-hydroxy-progesterone (104c)

Fig. 4 Inhibition of [3H] androstenedione binding in Sm cells by nonradioactive progesterone, androstenedione, cortisone, and triamcinolone (104d).

# INTRODUCTION

In assessing glucocorticoid activity, the common notion takes into account only two variables, free cortisol or corticosterone levels and the receptor status (capacity and affinity). I propose that though these are of obvious importance, they are not the only factors determining glucocorticoid impact on cells. The local activities of 11ß-dehydrogenase and 11-oxo-reductase and the circulating 11-oxy glucocorticoid levels are of importance as well. It follows that 1) a defective 11ß-HSD may cause glucocorticoid resistance or hypersensitivity, 2) the local activity and regulation of 11ß-HSD in individual tissues are of prime importance in determining the availability of active glucocorticoids to glucocorticoid receptors, and 3) cortisone and oxycorticosterone levels should be determined when evaluating glucocorticoid status. The present hypothesis expands the role of 11ß-HSD that was suggested by others. Thus 11ß-HSD can modulate cortisol availability not only in non-target tissues but also in target tissues of glucocorticoids. Further, 11ß-HSD can increase as well decrease cortisol availability to glucocorticoid receptors.

In this thesis,I will first review the literature on three related topics: a)11ß-Hydroxysteroid dehydrogenase (enzymology,biochemistry,regulation and physiologic significance),b) cortisol resistance and hypersensitivity.(cortisol resistance in humans squirrel monkeys,guinea pigs and in normal and malignant cell lines.A case of cortisol hypersensetivity in humans will be reviewed as well),and c) cortisone.(its concentration ,peripheral metabolism and significance as potential precursor for cortisol. Next, I will present my work in a format of three independent papers( each with its own introduction,materials and methods,results,,discussion and references). An overall summary and discussion wil follow. References for the introduction can be found at the end of the thesis.

In brief, this work was intended to study the following:

A. The regulation of 11ß-dehydrogenase and 11-oxo-reductase activities at the cellular and the molecular level. 1) Characterize their basal activities in several skin fibroblast cell lines in continuous culture (determine Km, Vmax, time course and substrate specificity). 2) Identify possible inducers or inhibitors of each activity and study their mechanisms of actions and their interactions. 3) Study the effect of induction/inhibition of both enzymes on the biologic effectiveness of cortisol and cortisone (in aromatase induction).

B. The possible involvement of these enzymes in the syndromes of cortisol resistance. 1) Determine their activities in skin fibroblasts from the squirrel monkey. 2) Modulate the resistance by enzyme inhibitors. 3) Possible models for involvement of 11ß-HSD in cortisol resistance include: increased 11ß-dehydrogenase activity ± decreased oxo-reductase activity; high affinity enzymes may compete with the glucocorticoid receptors for cortisol; they may be normally part of cortisol uptake mechanism but are dysfunctional in resistant cells; or they are normally part of an efflux mechanism and their activities are increased in resistant cells.

## LITERATURE REVIEW

A. 11ß-hydroxysteroid dehydrogenase (11ß-HSD)

11ß-hydroxysteroid dehydrogenase is a membrane bound (1) enzyme complex which catalyzes the reversible conversion between the biologically active C-11 hydroxyglucocorticoids (cortisol and corticosterone) and the biologically inactive (2) C-11 oxyglucocorticoids (cortisone and oxycorticosterone respectively). This enzyme complex is present in almost all tissues including uterus, gonads, liver, adrenal, brain, skin, intestine, stomach, pancreas, thymus, spleen, muscle, lung, kidney, thyroid, placenta (3) and adipose tissue (4).

Until recently the wide range of both the direction and the magnitude of this complex activity, reported in these different tissues, was accounted for by two distinct enzymes (11ß-dehydrogenase and 11-oxo-reductase) expressed variably in different tissues. Although several attempts to separate the oxidative activity from the reductive activity were unsuccessful, Lakshmi and Monder (1) provided strong, albeit circumstantial evidence for two separate enzymes.; 1) Detergents, phospholipases and elevated temperature increased the activity (released the latency) of the 11ß-dehydrogenase, while decreasing the activity of the 11-oxo-reductase, and the changes in activity were not related. 2) The oxo-reductase activity is permanently lost within ten minutes at 37°C while the dehydrogenase activity is stable for at least two hours. 3) The energy of activation determined from Arrhenius plots showed discontinuity at 23°C for the membrane-bound reductase but it was continuous for the membrane-bound dehydrogenase; both showed no discontinuity when released from the microsomal membrane by detergents or phospholipase treatment suggesting the presence of the two activities in different phospholipid environment. Further, the same group purified 11ß-dehydrogenase activity from rat liver microsomes using agarose-NADP affinity chromatography; the purified enzyme has no 11-oxo-reductase activity (5). Abramovitz, et at (6)

contrasting the activities in human fetal lung explants (dehydrogenase) with that of the monolayer cultures of the same tissue (oxo-reductive) reached the same conclusion. Moreover, there are several reported cases in humans, of patients with extremely low activity of the 11ß-dehydrogenase with intact 11-oxo-reductase (7) and of patients with extremely low 11-oxo-reductase and normal 11ß-dehydrogenase activities (8,9). It is clear now that one protein can catalyze both activities. Recent work by Schulz, et al (10) using isoelectric focusing analysis of detergent extracted rat renal 11-ß hydroxysteroid dehydrogenase showed a single peak at about pH 5.9 with both dehydrogenase and reductive activities at practically the identical position within the gel. Moreover, rat liver 11ß-HSD has been cloned and the expressed protein was endowed with both activities (11).

There may be more than one oxidase and more than one oxo-reductase. Monder and Lakshmi, studying rat liver microsomes, reported two kinetically distinguishable forms of the 11ß-dehydrogenase, based on curvilinear Eadie plots (Km of 2.3μM and 17.5μM for cortisol, and 0.09μM and 9.2μM for corticosterone)(12). However, their purified homogenous enzyme gave a rectilinear plot (Km of 17.30μM for cortisol and 1.86μM for corticosterone)(5). Another group also found only one 11ß-dehydrogenase in rat renal microsomal and nuclear fractions with a Km of $2.5 \times 10^{-7}$M in intact particulates (10) and between 1.8 to $6.3 \times 10^{-6}$M in extracts depending upon the detergent used (13). In human adipose tissue the kinetic studies of the 11ß-dehydrogenase are consistent with one enzyme with a Km of 0.5μM for cortisol (4). Finally, the human placental microsomal 11ß-dehydrogenase has a Km of 0.3μM for cortisol and the decidual 11ß-dehydrogenase has Km of 3.2μM (14). Similarly, Lakshmi, et al reported two Km values, for 11ß-oxo-reductase in intact rat liver microsomes of 0.72 and 7.7μM for cortisone (15). A detergent extracted 11-oxo-reductase from rat kidney has a

Km of $1.4 \times 10^{-6}$M to $4 \times 10^{-7}$M (for oxycorticosterone) (10). Human decidual 11-ß reductase has a Km of 0.3µM for cortisone (9).

Neither the 11-ß-dehydrogenase nor the 11-oxo-reductase are specific for their substrates. Even testosterone inhibits the decidual microsomal enzyme by 20%, when added in 10-fold excess of cortisol (14). 11ß-Hydroxy progesterone and its 20α and 20ß reduced derivatives are better competitors than cortisol itself for the human placental 11ß-dehydrogenase (16). Dexamethasone which is protected from the oxidation at C-11 by its 9-fluro substituent inhibited placental 11ß-dehydrogenase by 30% when added in 10-fold excess with cortisol (14). Conversely, 11-oxo-progesterone is a potent inhibitor of decidual 11-oxo-reductase (14).

It is clear that the activity of the 11ß-HSD complex is under tissue specific, developmental and hormonal regulation. In adults the liver and the lung express predominantly reductase activity, while the kidney, gonads and uterus are predominantly oxidative (3). In fetal mouse, the reduction/oxidation ratio in various tissues increases 5-50 fold between day 14 and day 19 of gestation (17). Similarly, the extremely high ratio of cortisone/cortisol metabolites in human fetal and newborn urine, reflecting high oxidative activity, changes to the adult value of 1.2 by one year of age (18). Cortisol increases mouse thymic cell 11ß-dehydrogenase activity 2-3 fold (19) and it also increases human fetal lung 11-oxo-reductase <u>in vitro</u> (20). Dexamethasone injection to 16-day pregnant mice resulted in an increase in the reduction/oxidation ratio in the placenta and fetal lung 16 hours later (17). Thyroid hormone excess in humans increases the overall oxidative and reductive activity, the former to a larger extent (21). Thyroxine adminstration, however, decreases the male rat liver dehydrogenase after seven days of exposure (22). In rats, a sex-dependent difference favoring males in the activity of the 11ß-dehydrogenase has been shown (22). Pepe, et al observed an alteration in

transplacental cortisol-cortisone interconversion from reduction at midgestation to oxidation at term in the baboon and attributed it to increased placental estrogens, since it was mimicked by treatment with the estrogen precursor, androstenedione, at midgestation (23). HCG induces human fetal lung 11-oxo-reductase in vitro (24).

Recently, several intriguing examples of the importance of the 11ß-dehydrogenase complex have been described

1) Murphy, et al (3) found that the 11ß-dehydrogenase activity was high in the placenta and fetal tissue, while the 11-oxo-reductase activity was high in the decidua and the uterus, and postulated that the inactivation of cortisol by the former protects the fetus against the growth-inhibiting effects of cortisol, while production of cortisol by the latter may contribute to the maintenance of the fetal allograft. Further, the general rise in the reductase activity of fetal tissues (and hence cortisol) close to term, is thought to be required for lung maturation.

2) Aldosterone receptors have equivalent affinity for aldosterone and glucocorticoids in vitro, yet, in vivo, only aldosterone is able to bind the aldosterone receptor in aldosterone target tissues(kidney,parotid glands,colon) despite the much higher circulating level of free glucocorticoids (25). An answer to this paradox has been suggested by the observations of high 11ß-dehydrogenase activity in aldosterone target tissues, but not in non-target tissues, and by eliminating aldosterone selectivity in vivo by treating the animals with the 11ß-dehydrogenase inhibitor ,carbenoxolone (26-29). Thus, the presence of 11ß-dehydrogenase activity in target tissues is believed to protect aldosterone receptors by converting glucocorticoids to their inactive metabolites. Aldosterone is protected from conversion by the enzyme owing to its 11,18 hemiketal or 11,18, 20 hemiacetal structure. This explanation is not entirely satisfactory, however, since the colon, a mineralcorticoid target tissue, has very little 11ß-dehydrogenase activity (20% conversion of 200,000 cpm of cortisol per 0.5g tissue at 37°C for 2

hours)(29) .11-Oxo-reductase activity was not measured in these experiments, although it is present in the kidney to a significant degree and occasionally is more active than the 11ß-dehydrogenase depending on the detergent used for extraction (10). Finally, the 11ß-dehydrogenase was localized to the proximal tubules both by measuring its enzymatic activity (8) and by immunohistochemistry (27), while the cortical collecting tubules are the aldosterone target tissues (30,31). The proximal tubules actually are the site of most of the glucocorticoid receptors in the mouse nephron (30).

3) A deficiency of the 11ß-dehydrogenase in the kidney has been suggested as the cause (7) of the apparent mineralocorticoid excess syndrome (32), where cortisol is free to activate renal aldosterone receptors causing sodium retention, hypokalemia, hypertension, and suppressed aldosterone as well as a decreased cortisol clearance rate. However, recently the same syndrome has been described with normal 11ß-dehydrogenase activity as reflected by the normal ratio of cortisol to cortisone metabolites in the urine (33). Also, several other aspects of the syndrome remain to be explained: the unresponsiveness to aldosterone infusion of up to 1 mg/day for five days (34); the decrease in cortisol metabolites not requiring 11-ß dehydrogenase (THF, αTHF, αcortol, ßcortol)(7,35); the relative deficiency of 5ß-compared to 5α–reduction and of 20ß-compared to 20α-reduction (7,35); the impaired conversion of tetrahydro to hexahydro steroids (35); and finally the successful treatment of this syndrome with dexamethasone (26), when dexamethasone has been shown to be a full aldosterone receptor agonist in that it is able to activate transcription of a reporter gene cotransfected with hmR in vitro (36).

4) A similar syndrome, caused by excessive licorice ingestion (37), or treatment with carbenoxolone, the synthetic derivative of its active component, glycyrrhetinic acid is also suggested to be due to inhibition of the 11ß-dehydrogenase in the kidney rather than, as once thought, to direct

mineralocorticoid activity of licorice (38, 39). This is because it has no effect in adrenalectomized animals and because glycyrrhetinic acid competes for aldosterone binding sites in kidney cytosol only at a concentration $10^4$ times that of unlabelled aldosterone (40), while glycyrrhetinic acid plasma levels in subjects consuming 100-200g of licorice per day is only 80-480 ng/ml (0.17-1μM)(41). However, in vivo, carbenoxolone decreased $^3H$ aldosterone binding to renal aldosterone receptors to 68% with the same dose which inhibited renal 11ß-dehydrogenase to about 50% (29). Also, carbenoxolone inhibits not only 11ß-dehydrogenase but also 11-oxo-reductase (41). Furthermore, although glycyrrhetinic acid has a Ki of $10^{-9}$-$10^{-8}$M for 11ß-dehydrogenase in rat kidney homogenate and microsomes, the Ki for intact tubules is around $10^{-5}$ (39), and there was only 17% inhibition (conversion decreased from 51.7% to 43.2%) of 11ß-dehydrogenase activity when the drug was given in vivo (39).

5) 11ß-dehydrogenase was localized to the Leydig cells in rat testes by immunfluorescence staining, and its appearance correlates temporally with the postnatal increase in Leydig cell numbers and the developmental rise in serum testosterone. The authors suggested that 11ß-dehydrogenase protects the testes from the deleterious effects of excess glucocorticoid (43). However, there was no determination of enzyme activity either of the 11ß-dehydrogenase or the 11-oxo-reductase. This is important, since as noted above, both activities might be due to the same or very similar proteins (10).

6) Recently a "late onset congenital adrenal hyperplasia like syndrome" has been described in a 28 year old female. Urinary steroid profiling showed that the ratio of cortisone to cortisol metabolites is 26 (normal, 1.5). Also, there was a markedly increased ratio of prednisone to prednisolone in the plasma and prednisone metabolites as compared to prednisolone metabolites in the urine when either was administered orally (8). A similar syndrome was also described in two

sisters, in whom the urinary steroid patterns revealed a virtual absence of 11ß-hydroxy metabolites (9). These data strongly suggest isolated 11-oxo-reductase deficiency as a cause of the syndrome.

B. Cortisol Resistance and Hypersensitivity

Both familial and acquired cortisol resistance has been reported in humans. Similar observations have been made in new world primates, guinea pigs and in normal and malignant cell lines.

1) In a Dutch family of 32 relatives, first described by Vingerhoeds et al (44), 4 members had the full syndrome of increased plasma free cortisol, increased urinary cortisol, normal circadian rhythm, apparent resistance to dexamethasone suppression, and absence of clinical stigmata of Cushing's syndrome during a follow up of more than 10 years. One member had hypertension and hypokalemia which were attributed to increased deoxycorticostrone and corticosterone secretion from the overstimulated adrenal glands. Both abnormalities were corrected by Dexamethasone treatment. CBG capacity and binding affinity were normal (46), and the serum free cortisol concentration was increased up to 6.5 fold. Glucocorticoid receptors, examined in whole cell assays using $^{3}$HDex as a tracer, had normal binding capacity and increased Kd in leukocytes (7.4 vs 2.8nM) and slightly increased binding capacity (170,000 sites/cell vs 133,000) and increased Kd (10.5 vs 6nM) in cultured skin fibroblasts (46). However, in the cytosol of broken cells the binding capacity of cytosolic receptor was decreased (26 vs 191 fmol/mg protein; 35 vs 95 fmol/mg protein, respectively) while the Kd was still increased (3.5nM vs 1.7; and 6.0nM vs 1.4 respectively). These results were interpreted as an "affinity mutant" as the primary defect together with receptor instability in broken cell preparations (46). Further studies with B-lymphocytes transformed with Ebstein Barr Virus also showed decreased binding capacity in

both whole cell (14,000 vs 28,000 sites/cell) and in cytosol preparations (90 vs 530 fmol/mg protein) and increased Kd (4.5nM, vs 1.3 and 8.7nM, vs 4.8 respectively)(47). The receptor was found to have a normal molecular weight (92 Kd) as determined by SDS-PAGE and nuclear binding was normal (47). An increased rate of dissociation of specifically bound ligand at elevated temperatures was observed, although the unliganded receptor was not less stable (47). Dexamethasone induction of aromatase activity in cultured skin fibroblasts, showed normal maximum stimulation; and a right shift in the concentration required for half maximum stimulation (5.7nM to 27nM)(47).

Although a decrease in receptor affinity may explain the apparent cortisol resistance, several questions remain to be answered. A 2-3 fold increase in the Kd would require only a 2-3 fold increase in the concentration of the free hormone, so that the same number of the receptors is occupied (pL=pT / 1+(Kd/LF) where pL is bound, LF is free hormone and pT is total binding sites). Thus a decrease in the affinity of this magnitude is not enough to explain the 6.5 fold increase in the free hormone concentration found in the index case. Further, an "affinity mutant" should show up as two binding sites on the Scatchard plot in the less affected member but this was not the case (46). Also, it is evident that there is a large discrepancy in the number of binding sites between the whole cell assay in the leukocytes and fibroblasts on one hand and the transformed ß lymphocytes and the cytosol of all three cell lines on the other (46, 47). Furthermore an altered affinity for Dexamethasone in a "mutant receptor" does not necessarily mean an altered affinity for cortisol. Unfortunately, no studies on cortisol metabolism were done, although a gas liquid chromatographic analysis of urinary steroids did show proportional increases in the THF and αTHE peak (44).

2) The same clinical syndrome was studied by Iida et al in Japan (49). Out of 8 family members examined the mother and a son, were found to have the

syndrome. Plasma free cortisol was 1.1 and 0.92 μg/dl (normal 0.27 ± 0.14), and Scatchard analysis of $^3H$-dexamethasone binding to peripheral leukocytes by a whole cell assay revealed a normal Kd of 2nm but a 55 to 65% decrease in binding sites (3.8 and 4.4 fmol/$10^6$ cells normal 7.2 ± 0.85) a 55 to 65% decrease (49). Since this may have been due to down regulation secondary to high cortisol levels rather than a primary defect, a follow up study in cultured skin fibroblasts (5-15 passages) was done, and confirmed a normal Kd of 3.75nM and decreased binding sites (6.96 and 7.86 fmol per mg DNA; normal 15.2)(50). Further, EBV-transformed lymphocytes also had a 50% decrease in receptor binding capacity. Labelling with $^3H$-Dex-mesylate showed an apparently normal 95 Kd receptor on SDS-PAGE. Furthermore, using an antibody against the c-terminal region of the rat GCR a western blot showed reduced intensity of a normal size band. A Northern blot showed a 7 Kd band with normal intensity and size and regions of mRNA corresponding to the DNA binding domain and steroid binding domain were normally protected in the patients and in the controls (51). These results were interpreted as a mutation in one GCR allele resulting in a truncated receptor which is unable to bind the steroid (51).

If the only abnormality in these patients is a 50% decrease in the binding capacity, then one would expect them to be hypoadrenal since there was only a 3.4 to 4 fold increase in the free hormone concentration. If normally 50% of the receptors are occupied, then to occupy the same number in the patient, all of the receptors of the patients should be occupied necessitating at least a 10-fold higher free hormone level (when $LF = 10Kd \rightarrow pL \approx pT$).

3) A new syndrome of adrenocortical micronodular dysplasia, cardiac myxomas and pigmented skin lesions was described in a family of three generations. Six of eleven evaluated members have two or three elements of the triad; of these, three have hypercortisolism as evident by increased 17-hydroxy

corticostroids, and increased urinary and plasma cortisol. All have resistance to Dexamethasone suppression. Interestingly only one has the physical finding of Cushing Syndrome, thus the other two are resistant to cortisol by definition. Surprisingly, Dexamethasone suppression resulted in paradoxical increase 17 OH CS (52). The pathologic diagnosis of adrenocortical micronodular dysplasia was made at autopsy in seven patients who did not have Cushing's syndrome, one of whom had massive intermittent hypercortisolemia (53). No studies of the cause of the apparent cortisol resistance in this syndrome were done.

4) A patient with adrenal adenoma and hypercortisolism without the characteristic features of Cushing's syndrome (excluding hypertension and polycythemia) was reported to have low GCR concentration 1370 sites per cell (normal 4850) in peripheral lymphocytes (54). In the same study there were no significant differences in the GCR concentration among healthy controls, 10 patients with Cushing's syndrome and three patients with Addison's disease (54).

5) Finally a thermolabile and activation labile GCR in cultured skin fibroblasts from a patient with the GC resistance syndrome has been reported in abstract form (55).

6) Pregnancy, and the somewhat related state, treatment with birth control pills are examples of acquired apparent cortisol resistance in humans. Since the 1950's it has been known that the plasma free 17-hydroxycorticoids are increased in pregnancy. This was shown later to be due mainly to elevated cortisol levels (56), due to the estrogen-induced rise in plasma CBG levels. More recent work has shown that both total and free cortisol are increased during pregnancy. The free cortisol index of 8 am serum samples in the third trimester determined by the charcoal adsorption method was 14.9 in the third trimester compared to 3.3 in nonpregnant women (57). Unbound cortisol determined by equilibrium dialysis was 0.058μM in the third trimester compared to 0.018 μM in the first trimester

(58). The increase in plasma free cortisol is also reflected by an increase in urinary free cortisol excretion (56,57). Resistance to cortisol is apparent because of the lack of clinical manifestations of Cushing's syndrome and by the gradual decrease of cortisol suppressibility with 1mg Dexamethasone overnight (OST) as pregnancy advances (free cortisol index:nonpregnant 0.4; first trimester 0.7, 2nd trimester 2, 3rd trimester 10.5.(58) Even after prolonged treatment with 2mg Dex/day for 7 days the mean free cortisol index was still $3.2 \pm 1.2$ (58). It is not likely that the increase in free cortisol is due to ectopic (placental) CRF or ACTH secretion since the diurnal pattern is intact in pregnancy (57,58) and the same phenomena is seen in women treated with birth control pills (60). It is also unlikely that the fetal adrenals are the source of the cortisol because of the diurnal pattern. Furthermore in two adrenalectomized pregnant patients maintained on 9-florocortisol during their 3rd trimester, no cortisol, cortisone, THF or THE was detected in the urine by sensitive assays (56). Also, in the urine of an Addisonian 3rd trimester pregnant patient no cortisol or cortisone and only 50μg each of THE and THF were detected (61). The plasma free cortisol determined by equilibrium dialysis at 9-9:30 am, on day 18 in 15 normal cycling women and in 15 women taking 30μg ethinyl estradiol and 150μg desogestrel for at least 6 months, were $18.0 \pm 7.95$ and $32.3 \pm 9$nM, respectively (60). The salivary cortisol, a reflection of plasma free cortisol, were also increased from $9.2 \pm 3.9$ to $18.8 \pm 6.9$ (60). As expected there was no signs of Cushing's syndrome. It was proposed that the apparent cortisol resistance is due to the progestin component of the pill and the increasing levels of progesterone during pregnancy (60); however, several observations are not consistent with this:

(1) If KL and LF are the dissociation constant and the free concentration for cortisol, and KI and IF are those for progesterone, then the GCR bound to cortisol is pl = pT /1+KL/LF + KL IF/KI LF) in this example pT, KL and KI are constant and to get the same binding (pL) the ratio IF/LF should be constant. An increase in

free progesterone from 0.0039μM in the first trimester to 0.0069μM in the third trimester (1.77 fold)(58), would then require only 1.77 fold increase in the free cortisol; however the free cortisol is increased from 0.018 to 0.058μM (3.2 fold)(58). In other words, suppose KL=50nM and KI=5000nM then in the first trimester pL = pT/3.78 and in the third trimester pl = pT/1.86 or 2 folds increase in the glucocorticoid binding and hence effect, which is not seen. (if KL=100 and KI=10,000 then there will be 2.4 fold increase, if KL=50 and KI=500 then there will be 2.03 fold increase, if KL=50 and KI=5,000 then there will also be 2.03 fold increase).

(2) The same syndrome is seen in women given ethinyl estradiol or diethylstilbesterol without any progestin (62,63) and also in men treated with estrogens (63,64).

(3) Of note is the altered cortisol metabolism seen in these conditions. In pregnancy, the half life of plasma free 17-OH-corticostroid is doubled (56) and that of cortisol is increased from 69.5 minutes to 104.6 minutes (57). Despite the increase in plasma cortisol and urinary free cortisol (1.79 to 3.70), urinary free cortisone showed little change (0.65 to 0.86) and the combined THF + THE (there were no reported individual values) decreased from 21.63 to 16.06 mg/24 h There was no change in total urinary 17-hydroxy corticosterone (56). In normal men and women treated with 0.5mg ethinyl estradiol per day for 2-3 weeks, there was also a decrease in a urinary 17-hydroxy corticosterone (30-50%) with no change in 17-keto steroid excretion (63). Although the plasma cortisol half life more than doubled, the half life of cortisone, DHF and THF did not change (63). Finally, in the group taking the combined ethinyl estradiol and desogestrel there was almost a two fold increase in the salivary to plasma free cortisone ratio (2.3 to 4.4) suggesting an increase in 11-ß dehydrogenase activity in the salivary glands (60).

7) Anorexia nervosa is another example of acquired cortisol resistance. This syndrome is characterized by distorted body image, obsessive dieting, weight loss, physical hyperactivity, amenorrhea and several endocrine abnormalities. The early morning total plasma cortisol is increased 2 fold (23.3 vs 10 μg/dl )(65). The total binding capacity of CBG in one study was slightly higher than normal (29.9 vs 21.9μg/dl) but the difference was not statistically significant. However, the affinity constant for cortisol measured at 0°C was lower ($14.1 \times 10^8 M^{-1}$ vs $22.3 \times 10^8 M^{-1}$)(65). As expected, the free plasma cortisol, measured by equilibrium dialysis at 8 am was higher than normal (15 vs 8ng/ml)(66). The circadian rhythm is maintained in these patients (66,67), but there is an impaired response to the overnight dexamethasone suppression test (66). These findings together with the absence of the stigmata of Cushing's syndrome, again define a state of cortisol resistance. The half life of cortisol is prolonged from 60 to 78 minutes and the MCR is decreased from 359 to 177 liters per day (67). Cortisol production rate was normal in one study (67), and elevated (24.3 vs 18mg/24h) in another (68). In general, these abnormalities correlate with percent loss of ideal body weight (66) and tend to improve upon weight gain (65). The cause of the cortisol resistance in this syndrome is not clear. Female patients are amenorrheic and therefore progesterone is absent from the plasma (64). Because these patients usually have low T3 secondary to decreased deiodinase activity, it was postulated that the low cortisol MCR is due to a "hypothyroid state", especially since treatment with T3 improved the MCR toward normal (67). However, whether these patients are really hypothyroid is not clear, since their TSH levels were normal (66). Further, the treatment resulted in plasma T3 concentrations several fold above normal and there is no data on plasma cortisol levels after treatment (67). Furthermore, patients with myxedema have normal plasma cortisol concentrations (69). One group studied the GCR in the mononuclear cells from these patients in a whole cell assay

using $^3H$ Dex as tracer and found a normal Kd (8.4 vs 11.4nM) but a decreased number of binding sites ($3.8x10^3$ vs $4.9x10^3$ sites/cell)(70). This may be a primary defect, since patients with Cushing syndrome with even a higher degree of hypercortisolism have normal receptor binding capacity (54). Interestingly, despite increased urinary free cortisol (225 vs 65μg/day) there is decreased excretion of cortisol metabolites (THE, αTHE, THF, αTHF) in the urine at basal state and during ACTH stimulation (2.8 vs 3.4mg/24h; and 11.8 vs 23mg/24h respectively); there also is a relative deficiency in 5α reduction since (the ratio of etiocholanolone and its 11-OH and 11-Oxy derivatives to their respective 5α homologus was 1.9 rather than 1.0 (71).

8) New world primates (e.g., squirrel monkey, marmoset, and the owl monkey) are well known for their resistance to cortisol and other steroids. In the squirrel monkey total plasma cortisol is increased some 26 fold (199μg/dl as compared to 11.5 in man, an old world primate; and to 5.7 in Lemurinae, a prosimian)(72). The binding capacity of CBG is decreased 10 fold (2.3μg/dl vs 22.2μg/dl in man) and its affinity (Kd) for cortisol is reduced (at 37°C $0.5x10^7$ vs $4.8 \times 10^7$L/Mol in man)(73). This results in a 100 fold increase in free cortisol (30.6vs 0.29 in man), and in urinary free cortisol (129 vs 0.7 μg/kg/24h, in man)(72). This striking level of free cortisol is maintained by a 6 fold increase in cortisol production (mild adrenal enlargement, hypertrophy of the zona fasiculata, relative increase in 21 and 11 hydroxylase activity, and relative decrease in 17-20 desmolase activity) and by 2 fold decrease in cortisol MCR (74). In the squirrel monkey, plasma cortisol is resistant to suppression by overnight dexamethasone, as it requires 46 fold more Dex to achieve 50% decrease in plasma cortisol levels (72). This might be due in part to the much higher initial values combined with the decreased MCR of cortisol. The lack of stigmata of Cushing's syndrome, the resistance to Dex suppression and the extraordinary free cortisol, define the cortisol

end-organ resistance syndrome. The squirrel monkey GCR has been examined in circulating leukocytes and cultured skin fibroblasts and compared with that studied in human cells (72). The binding capacity of leukocyte GCR was $6.4x10^3$ sites/cell vs $3.8x10^3$ in man and in fibroblasts, $94x10^3$ sites/cell vs $133x10^3$ in man. The Kd for Dex binding was 49nM vs 2.2 in man and 13nM vs 6nM in man, respectively. However, transformed marmoset lymphocytes showed an increased Kd and a large decrease in binding capacity (7 vs 40 fmol/$10^6$cells in human in a whole cell assay and 50 vs $\bar{6}00$fmol/mg protein in human in a cytosol assay (76). The receptor from the transformed cells had a normal molecular weight of about 92Kd on SDS-PAGE, normal nuclear translocation, and normal thermal activation as determined by phosphocellulose retention. However, there was a greater loss of specifically bound ligand during thermal activation which was not due to receptor degradation (76). The investigators suggested that a mutant receptor, with decreased affinity for cortisol is the primary event, leading to compensatory increase in the circulating cortisol to maintain a normal glucocorticoid status. They suggested that the loss of specifically bound ligand upon receptor activation is secondary to this decrease in affinity (76).

It is not clear then, why the free cortisol is increased 100 fold. The Kd was increased only 2 fold in the skin fibroblasts and 22 folds in the circulating leukocytes, as discussed before, this will require only a 22 fold increase in the circulating free cortisol. There is also no clear explanation for the striking difference in the receptor Kd in different cells and the discrepancy in the binding sites between normal and transformed cells. In this regard, a whole cell assay for GCR in our laboratory revealed a normal Kd and a 50% decrease in binding sites as compared to human cultured skin fibroblasts. Further, one has to postulate a second mutation affecting the capacity and the binding affinity of CBG. Finally, of most interest is the observation that cortisol in the squirrel monkey, doesn't have

the expected mineralcorticoid effect (75). Renal aldosterone receptors in the squirrel monkey have normal affinity for aldosterone 22$\pm$14 vs 16nM in the cynomolgus monkey in adrenalectomized animals, and 43$\pm$15 vs 17nM in intact animals. However, the Ki of cortisol for aldosterone binding is 27 fold higher in the squirrel monkey ($7.8 \times 10^{-7}$ vs $2.9 \times 10^{-8}$) than in the cynomolgus)(75). Although not examined directly, it appears that progesterone also has lower affinity for the aldosterone receptor; since despite a 20 fold increase of the antagonist progesterone during pregnancy (8 ng/ml to 140-490), there is no change in plasma aldosterone (30$\pm$18 ng/dl to 38+8)(75).

9) In the guinea pig the mean circulating cortisol level is $16 \times 10^{-7}$M, which is about 4 fold higher than in humans ($4 \times 10^{-7}$M)(77). CBG levels are roughly the same as in humans, but the protein has lower affinity for cortisol (Kd $2 \times 10^{-8}$M vs $1.7 \times 10^{-9}$M)(76) resulting in a much higher free glucocorticoid level compared to those found in humans or rats. Resistance is further demonstrated, by the fact that the glucocorticoids concentration required for half maximal inhibition of thymidine incorporation in phytohemagglutinine stimulated spleen cells is 80mM in the guinea pig as compared to 5nM in the mouse (78). The GCR was examined in guinea pig kidney and liver cytosolic preparation and found to have normal capacity but lower affinity for dexamethasone ($2 \times 10^{-7}$M vs $0.3\text{-}4.2 \times 10^{-8}$M in humans)(77). Further, it was found that these cytosolic preparations possess an inhibitory activity, as reflected by inhibition of dexamethasone binding to GCR rat liver cytosolic preparation. This activity is diminished by previous adrenalectomy, is not a steroid, is not removed by charcoal extraction and can exert its effect across the cell membrane. It is postulated to be a metabolizing enzyme (77).

10) In vitro, resistance to GC has been studied in lymphocytes (lysis) and in fibroblasts (growth inhibition). Recent work has emphasized the role of GCR. Thus in cultured lymphoma cell lines resistant to GC, 80% were found to be GCR

deficient, 10% deficient in nuclear transfer and 10% deficient in reactions subsequent to nuclear transfer (79). In CEM cells, a model for childhood acute lymphocytic leukemia, four types of resistance have been identified; pseudo-resistance (prolonged time required for the effect to take place); DNA hypermethylation (sensitivity is recovered by 5-aza-deoxycytidine, which interfers with cytidine methylation); a decrease in receptor sites; reduced nuclear transfer and activation labile receptors (lost ability to bind steroid upon activation)(80). However, it has been known since the 1960(s) that malignant lymphocytes have a greater capacity to inactivate cortisol than normal lymphocytes (19). Similarly, fibroblasts sensitive to the growth inhibition of cortisol in tissue culture, actively transform cortisol (81), although the correlation doesn't always exist (82). Of note, is a study comparing lymphoma cell which are corisol resistant, to normal lymphocytes in regard to their 11-ß dehydrogenase activity. At 5 and 15 minutes, there was much more reductase activity in the lymphoma cells, and much more oxidase activity in the normal cells. However, at 180 minutes the lymphoma oxidase surpassed that of the normal cells, and at 60 minutes the normal cells oxidase surpassed that of the lymphoma cells. The authors suggested that this is due to different permeability of cortisol and cortisone in these cell lines (82).

11) Interestingly, there exist not only natural examples of cortisol hyposensitivity but also of cortisol hypersensitivity. A patient with the clinical picture of Cushing's syndrome and hypocortisolemia recently has been reported in abstract form. He has low plasma cortisol, low urinary free cortisol and low urinary 17 hydroxy corticosteroids, even after 6 months of careful observation during hospitalization. He has low plasma ACTH levels , but a normal cortisol response to ACTH. The capacity and binding affinity of his CBG are normal. His cultured skin fibroblasts are hypersensitive to GC as determined by inhibition of thymidine incorporation. The affinity of the GCR to Dex is normal in leukocytes,

cultured skin fibroblasts and in Ebstein-Barr-transformed lymphocytes. However, the affinity of the GCR from the same cells for cortisol is lower (83-84). This certainly suggests involvement of a metabolizing enzyme or a carrier more specific for cortisol than for Dex.

### C. Cortisone - The Neglected Precursor

The ratio of circulating cortisone to circulating cortisol varies with age. In the newborn, the mean concentration of cortisone is 83+11.2μg/L and of cortisol is 104.3+25.7μg/L; in a 7 day-old-infant it is 22+4.6μg/L and 34.8+15.7μg/L respectively (85). In normal adults, the median concentration of cortisone is 18μg/L (6-24) and of cortisol is 80μg/L (50-160), as measured by HPLC between 0800 and 0900 h (86). Thus the cortisone to cortisol ratio in adults is 1 to 5-10 but it is much higher in infants. However, since cortisol binds avidly to CBG, only 5-10% of the cortisol, but most of the cortisone is free to enter cells; so that there are equal amounts of available cortisol and cortisone. This has been confirmed using equilibrium dialysis (60), and by the finding of similar excretion rates of free cortisol and cortisone in the urine (6.7μmol/mol creatinine, and 8.0μmol/mol creatinine respectively)(87).

The maximal concentrations of free cortisol are around the Kd for the GCR and hence are on the linear part of the saturation curve. On the other hand the biologic effects of steroid hormones are directly proportional to receptor occupancy (78) hence are very sensitive to changes in the ambient concentration of cortisol.

The liver is the principle organ that produces steroid-sulphate conjugates and glucuronide conjugates although the kidney and adrenals can make small amounts. Tetrahydro cortisol in which the 4-5 double bond is saturated and the 3-keto group is reduced to a 3-hydroxy group is a major product of hepatic metabolism. However, extrahepatic tissues can carry out some of the oxidation and

reduction reactions performed by the liver at C-11 and C-20 (88). Fibroblasts have been shown to convert cortisol to 20α-and 20ß-cortisol, 20α- and 20ß-cortisone, cortisone, dihydrocortisol and 11ß-androstenedione (89). Fibroblasts are also able to convert cortisone to cortisol (88). Moreover, cells in tissue culture have been shown to respond to cortisone. In human fetal lung cell culture, it was observed that not only cortisol but also cortisone, enhanced growth though to lesser extent (20). The cells were found to convert cortisone to cortisol (20). Recently, cortisone was shown to reduce nerve growth factor levels and its mRNA in fibroblasts conditioned medium, with 50% inhibition occurring at a concentration of $5x10^{-9}M$ (90).

With these facts in mind, it is surprising that circulating cortisone is ignored when assessing glucocorticoid status. Further, it follows that the circulating free cortisol is not necessarily a reflection of the available intracellular cortisol. For example, a free concentration of cortisol and cortisone of 20nM and a 50% conversion of cortisone to cortisol with no conversion in the opposite direction will increase intracellular cortisol to 30nM; a 50% conversion of cortisol to cortisone with no conversion in the opposite direction will decrease intracellular cortisol to 10nM; a 3 fold difference. Thus individual cells may have the ability to fine tune their glucocorticoid status. Obviously this depends not only on the circulating cortisol and cortisone levels, but also on the activity of the 11ß-dehydrogenase and 11-oxo-reductase.

Regulation of 11ß-Hydroxysteroid dehydrogenase (11ß-HSD) activity in human skin fibroblasts: Enzymatic modulation of glucocorticoid action*.

Muhammad M. Hammami and Pentti K. Siiteri.+

From

Reproductive Endocrinology Center

University of California, San Francisco

San Francisco, Ca. 94143

Running title:

-

**11ß-HYDROXY STEROID DEHYDROGENASE AND GLUCOCORTICOID ACTION**

## SUMMARY

The regulation of 11ß-hydroxysteroid dehydrogenase (11ß-HSD)[1] was studied in cultured human skin fibroblasts. 11Oxo-reductase activity was 5-10 fold higher than 11ß-dehydrogenase activity. Cells treated with 100nM Dexamethasone (Dex) showed a three fold increase in Vmax of both activities without a change in Km values. Dex induction of 11β-HSD was half maximal at 48 hours and was blocked by glucocorticoid receptor antagonists. Non-glucocorticoid steroids were ineffective. Removal of serum from the culture medium increased Vmax values up to 6-fold. Treatment of cells grown in the absence of serum with 8-bromo-cAMP, phorbol esters or insulin decreased both11ß-HSD activities. The effects of Dex treatment and serum removal were additive and were blocked by cycloheximide and actinomycin D. In all experiments both 11ß-HSD activities were modulated in parallel. Both cortisone (200nM) and cortisol increased aromatase activity of fibroblasts in the presence of serum. Prior induction of 11β-HSD by serum removal increased the potency of cortisone from 10-15% to 50% that of cortisol.

We conclude that, 1) in fibroblasts 11ß-HSD is a single protein that is under multifactorial regulation 2) 11β-HSD may increase or decrease cortisol availability to glucocorticoid receptors and, 3) plasma cortisone levels may be important in assessing glucocorticoid status.

Although 11β-HSD is present in almost all tissues (1-2) its physiologic significance is still largely unknown. Recently, it was proposed that the presence of 11ß-dehydrogenase activity in aldosterone target tissues serves to confer specificity to aldosterone receptors by conversion of cortisol, or corticosterone, to their corresponding 11 keto steriods which have low affinity for glucocorticoid receptors (3-7). If true, this could explain the paradoxical findings that aldosterone and cortisol display similar affinities for the aldosterone receptor *in vitro* (3,8,9), whereas aldosterone receptors bind aldostrone selectively *in vivo* (7), even though the circulating free levels of cortisol are 10-100 fold higher than those of aldosterone (3). However, the following observations are not consistent with this interpretation; 1) very low 11β-HSD activity is found in the colon, an important mineralocorticoid target tissue (7), and 2) 11β-HSD was localized to the renal proximal tubules (5,10), the site of most of the glucocorticoid receptors in the nephron, rather than the cortical collecting tubules where aldosterone receptors are localized (10,11).

The ratio of total cortisone to cortisol in human plasma decreases from 0.8 in the newborn to 0.2 in adults (12,13). However, the concentrations of free cortisol and cortisone in adult plasma are about equal owing to the fact the 90% of the cortisol but very little cortisone is bound to corticosteroid binding globulin (14). Circulating cortisone therefore represents a large precursor pool that may augment glucocorticoid action in target cells containing 11β-HSD.

We have characterized the basal 11ß-dehydrogenase and 11oxo-reductase activities of 11β-HSD in cultured human skin fibroblasts and have identified positive and negative regulators of the enzyme. We found that glucocorticoids acting through the glucocorticoid receptor increased 11β-HSD activity, whereas

addition of serum, insulin, cAMP or phorbol esters decreased 11β-HSD activity. 11ß-Dehydrogenase and 11oxo-reductase activities changed in parallel fashion under all conditions, strongly suggesting that a single protein catalyzes both reactions. Finally, we demonstrated enhanced induction of the aromatase enzyme in human skin fibroblasts by cortisone following treatments that increased 11β-HSD activity.

# MATERIALS AND METHODS

## MATERIALS

Dulbecco's modified Eagle's medium (DME) (4.5g/L glucose, 0.584g/l L-Glutamine, 3.7g/L NaHC03) free of phenol; fungizone 250µg/ml; penicillin G 10,000 units/ml and streptomycin sulfate 10,000 µg/ml; insulin 1mg/ml (24u/mg); and calf serum were obtained from the tissue culture facility of University of California, San Francisco. Six well plates were obtained from Becton Dickinson Labware, Lincoln Park, New Jersey. [1,2,$^3$H] cortisone and [1,2,6,7,$^3$H] cortisol were obtained from Amersham, Arlington Heights, Illinois and New England Nuclear (NEN) Boston, MA respectively; nonradioactive steroids were from Steraloids Inc., Wilton, NH; RU486 and dexamethasone mesylate from NEN; Actinomycin D, cyclohexamide, human albumin (essentially fatty acid and globulin free) fibroblast growth factor (FGF), epidermol growth factor (EGF) and 8-bromo-cAMP (cAMP) were obtained from Sigma, St. Louis, MO. Normal human adult foreskin fibroblasts (HS681) were obtained from the tissue culture facility of University of California, San Francisco, CA. Human adipocyte stromal cell cultures were prepared from liposuction specimens by collagenase treatment. Thin layer chromatography plates coated with silica gel were obtained from Whatman Ltd. Kent, England.

**METHODS**

Cells were maintained in DME (phenol free) medium supplemented with 10% calf serum (CS), penicillin (100 units/ml) and streptomycin (100µg/ml), amphotericin β (Fungizone; 2.5µg/ml) and fibroblast growth factor (2ng/ml). Cells were plated in 6-well plates and grown until confluent or near confluent. After washing three times with DME, cells were incubated with substrates and/or effectors for the indicated times with or without additions.

Tritium labelled cortisol and cortisone (specific activities of 99.8 and 29ci/mmol, respectively) were purified by celite column chromatography prior to use. The solvent systems were iso-octane: t-butanol: $H_20$ (250: 125: 225) for cortisol and (100: 35: 90) for cortisone. The eluted peaks were collected, dried and reconstituted in ethanol and kept at $4^o$. After purification, [$^3$H] cortisone contained less than 1% cortisol and [$^3$H] cortisol less than 2% cortisone as determined by thin layer chromatography (TLC). The data points were corrected for the amount of cross contamination as determined in each experiment.

Enzyme assays: monolayers of cells in 6-well plates were incubated with radioactive substrate in DME at $37^o$ (CO2 5%, air 95%) for the indicated times. An aliquot of the medium was then extracted with 10 volumes of ethyl acetate. The ethyl acetate extract was evaporated under a stream of nitrogen and the radioactivity was reconstituted with 100µl ethyl acetate containing cortisol and cortisone standards (1mg/ml). Duplicate aliquots were applied to TLC plates and another aliquot was counted to monitor recovery. TLC plates were developed in chloroform: ethanol: t butanol (9:1:1), dried and the standard spots visualized by first spraying with a mixture of glacial acetic acid, $H_2SO_4$(conc.) and p-

anisaldehyde (50:1:1) and then heating in a vacuum oven at 100° for 5-10 minutes. The identified spots were cut out, eluted with 0.3ml of ethanol into mini-vials and the tritium counted after adding scintillation fluid. In preliminary experiments it was found that the cortisol and cortisone spots accounted for more than 95% of the total radioactivity recovered from the plates. Negligible radioactivity was associated with 20α- or 20β- reduced cortisone which were clearly separated from the cortisol standard. The recovery of total radioactivity applied to the TLC plates averaged 80% and did not vary significantly between the controls (no cells) and the experimental samples. Cell layers, washed twice with DME, were solubilized by incubating overnight with 0.5N NaOH at room temperature and cellular protein (about 300μg per well) was determined by the Bradford assay (15). Results are expressed as pmol/mg protein/h of the product formed and were calculated by multiplying the percentage of substrate conversion by the initial amount of substrate and then adjusting for protein and time of incubation. For kinetic studies, the data were linearized according to Eadie-Scatchard analysis (V/S=Vmax/Km-V/Km, where V is the rate in pm/mg protein/h and S is the substrate concentration in nM). A least squares best fit computer program was used to calculate the Km and Vmax values.

Stock solutions of steroids and drugs prepared in ethanol, and stored at 4°, were evaporated under nitrogen and reconstituted in DME before being added to cells. Serum was treated twice with dextran-coated charcoal (DCC) for 1 1/2 h at 37° to remove endogenous steroids. Aromatase activity was measured as previously described (16).

**RESULTS**

Basal 11oxo-reductase and 11ß-dehydrogenase activities in human genital skin fibroblasts: Using several concentrations of cortisone as substrate, the reductase activity was determined at intervals up to five hours. The data obtained using substrate concentration of 995nM, is presented in Fig. 1 (a). As shown, the reductase activity was linear between 10 minutes and five hours. All further experiments were performed within this time period. Fig. 1 (b) shows substrate saturation of the reductase activity determined at ten minutes. The apparent Km and Vmax values obtained in this experiment are 219nM and 20pmol/mg protein/h, respectively. The 11ß-dehydrogenase activity was much lower and could not be detected at 10 minutes. 11ß-Dehydrogenase activity using 300nM cortisol as substrate is shown in Fig. 1 (c) (control). Since oxidation of cortisol was much slower, 11ß-dehydrogenase activity could be underestimated due to rapid back conversion of cortisone to cortisol. This was demonstrated by addition of non-radioactive cortisone together with radioactive cortisol substrate and as expected, the measured oxidation rate was increased Fig. 1 (c) (+ cortisone). Therefore, all subsequent 11ß-dehydrogenase determinations were performed in the presence of unlabelled cortisone at 100-1000 fold excess of [$^3$H] cortisol. Fig. 1 (d) shows substrate saturation of the oxidation reaction measured at one hour in the presence of 100 fold unlabelled cortisone. This data yielded a Km of 220nM and a Vmax of 4pmol/mg protein/h.

Glucocorticoid induction of 11ß-HSD activity: Possible regulation of 11ß-HSD activities by steroid and thyroid hormones was examined by preincubating the cells for 18 or 72 hrs with the following: Dex (100nM), estradiol (50nM), progesterone (5µm), testosterone (500nM), and triiodothyronin (2nM) in medium containing 10% charcoal stripped calf serum (SCS). As shown in Fig. 2 (a) only Dex treatment increased the 11oxo-reductase activity 2-3 fold. Interestingly, 11ß-

dehydrogenase activity also was increased only by (Dex Fig. 2 (b)). To further characterize Dex induction of 11oxo-reductase activity, cells were preincubated with 100nM Dex in medium containing 10% SCS for 18 to 170 hrs. The Dex effect was apparent at 18 hrs, half maximal at about 48 hrs and maximal (4.5 fold) at 7 days (Fig.3 (a) and data not shown). Fig. 3 (b) shows that half maximal induction was produced by about 20nM Dex, which is consistent with its affinity for the glucocorticoid receptors in human fibroblasts (in these cells there are 123 x $10^3$ Dex binding sites/cell with a Kd of 5-10nM). We also examined the effect of cortisol treatment with and without the glucocorticoid receptor antagonists, RU486 and Dex mesylate on 11β-HSD. Table 1 shows that half maximal induction by cortisol occurred at 200nM and that much higher levels were required when the antagonists were added, indicating that glucocorticoid effects are mediated by the glucocorticoid receptor. Furthermore, addition of cycloheximide (0.5μg/ml) or actinomycin D (0.005μg/ml) to cells decreased 11β-HSD induction by 20μM cortisol to 20% and 30% of control respectively, suggesting that protein and mRNA synthesis are required for glucocorticoid stimulation of 11β-HSD.

The inhibitory activity of serum on 11β-HSD: We tested for other possible regulator(s) of 11β-HSD by growing cells in the absence of serum for various time periods. Removal of serum from the growth medium caused a significant increase in both the 11ß-dehydrogenase and the 11oxo-reductase activities of fibroblasts. This was evident even without correcting the rates of conversion for the decrease in protein concentration caused by removal of serum. Fig. 4 (a) shows the time course of 11oxo-reductase activity following replacement of serum with 4mg/ml human albumin. The 11β-HSD activity increased up to 8 fold 48 to 72 hrs after serum removal. When different amounts of human serum were added back to cells the 11oxo-reductase activity decreased as shown in Fig.

4(b). The decrease in 11oxo-reductase activity was half maximal with 2-5% human serum, while 10-15% serum caused maximal suppression. Squirrel and Rhesus monkey sera gave similar results. Calf serum was moderately less active and its inhibitory activity varied slightly between batches. Stripping the serum with charcoal did not significantly affect its inhibitory activity. Addition of cyclohexamide or actinomycin D reduced the effect of serum removal on 11β-HSD.

We examined various hormones and potential second messengers to identify constituents of serum responsible for its repressive effect on 11β-HSD. Fig. 5 shows that addition of human insulin to serum free medium was effective with half maximal inhibition observed at 1-10 nM. Similarly, addition of insulin blocked induction of the 11oxo-reductase activity by Dex (Table 2 (a)). Furthermore, insulin also decreased 11β-dehydrogenase activity of cells maintained in the presence of 100nM Dex or in serum free medium (Table 2(b)). Addition of the growth factors FGF (100ng/ml) or EGF (100ng/ml) lowered the 11oxo-reductase activity of cells maintained in serum free medium by only 20% and 38% respectively. Furthermore, addition of 8-bromo-cAMP (cAMP) or phorbol myristate acetate (PMA), resulted in moderate inhibition of the serum removal effect (data not shown).

11Oxo-reductase and 11ß-dehydrogenase activities are regulated in parallel: As shown in Fig. 2, Dex induces both 11ß-HSD activities to a similar extent. Table 2(c) shows the effect of 20μm cortisol or 20μm cortisone added to cells in the presence or absence of 10% SCS on both activities. The results indicate that 1) the effects of glucocorticoids and serum removal are additive or weakly synergistic, and 2) that treatment with either steroid increases 11ß-dehydrogenase and 11oxo-reductase activities to a similar extent. Cortisone, which is considered to be an

inactive glucocorticoid, apparently is effective in inducing both activities by being converted to cortisol. We also observed similar additive effects of glucocorticord treatment and serum removal with parallel increases in both 11β-HSD activities in primary cultures of human adipose stromal cells, although Dex treatment was more potent than serum removal (4-5 fold and 2-3 fold increases, respectively). Table 3 summarizes the results obtained from Eadie-Scatchard plots of both basal and induced 11oxo-reductase and 11ß-dehydrogenase activities. The increase in both activities is due to an increase in Vmax without any significant change in Km, further indicating that these treatments induce new enzyme synthesis. Moreover, the parallel changes in both 11β-HSD activities clearly indicates that they are catalyzed by one rather than two different proteins.

We also examined the effects of adding the product cortisol on the 11oxo-reductase assay, and the effects of the inhibitors, 11β-hydroxy progesterone and 11 –oxy progesterone (17,18) on the 11oxo-reductase and 11ß-dehydrogenase activities, respectively (data not shown). Addition of cortisol or 11ß-hydroxy progesterone inhibits the 11oxo-reductase activity as expected by product inhibition. Moreover, the augmentation of the 11ß-dehydrogenase rate by addition of the product cortisone can be mimicked, though to a lesser extent, by the 11oxo-reductase inhibitor 11-oxy-progesterone. However, when the amounts of added cortisone or 11-oxy-progesterone were increased beyond 1000 fold excess, product inhibition predominated and the 11ß-dehydrogenase activity decreased (data not shown).

Cortisone induction of aromatase activity: The high 11oxo-reductase activity observed in fibroblasts together with the results shown in Fig. 6 indicated that cortisone may be activated in peripheral tissues without the need for conversion to

cortisol in the liver, as is commonly held. To test this notion we measured fibroblast aromatase activity following addition of 200nM cortisol or cortisone. The results are shown in Fig. 6. Cortisone was 17% and 34% as effective as cortisol, in aromatase induction at 6.5 h and 17 h, respectively. The effect of the increased 11β-HSD activity following serum removal on aromatase induction by cortisone was also examined. The accelerated conversion of cortisone to cortisol resulted in an apparent increase in cortisone potency (6.7 fold) up to 50% of that observed with cortisol.

## DISCUSSION

11β-HSD is a membrane bound pyridine nucleotide (NADP-NADPH) dependent enzyme complex (19-21) that catalyzes the interconversion between the biologically active 11ß-hydroxy glucocorticoids and their biologically inactive 11-oxoglucocorticoid counterparts (22). We have characterized the basal activities and regulation of 11ß-HSD in cultured normal human genital skin fibroblasts, that are known target cells for glucocorticoids.

Human fibroblasts have been shown to carry out oxidation and reduction at both C-11 and C-20 of glucocorticoids (23). In the present system, there is little activity, if any, at C-20 since cortisol and cortisone accounted for more than 95% of steroids recovered from the TLC plates, even after incubation up to 20 hours. The estimated Km values for the 11oxo-reductase and 11β-dehydrogenase activities measured in intact fibroblasts are in accord with values reported in other studies of human tissue homogenates (2, 17). The Km values of 200-300nM found with whole cells in this study are about equal or 10 fold higher than the concentrations of circulating cortisol and cortisone, respectively. Consequently, 11β-HSD can

produce significant changes in intracellular cortisol and cortisone levels under various physiologic and pathologic conditions that alter the redox state of cells.

Previous studies have suggested that 11β-HSD is under complex hormonal regulation (24-30). We found that the active glucocorticoids, cortisol and Dex, increase both 11β-dehydrogenase and 11oxo-reductase activities in human fibroblasts. This effect is specific, since other steroid hormones at their biologically maximum effective concentrations, were inactive. Further, the increased enzyme activity following glucocorticoid treatment is likely mediated by glucocorticoid receptors since dose response curves correlated well with affinities for the receptor. Furthermore, the effect of cortisol was greatly diminished by the glucocorticoid receptor antagonists, RU486 and Dex mesylate. Moreover, the glucocorticoid stimulatory effect on 11ß-HSD was shown to be dependent on mRNA and protein synthesis. Taken together these data strongly suggest that glucocorticoid treatment induces increased enzyme synthesis by increasing the rate of transcription of the 11β-HSD gene. However, whether this effect is a primary or a secondary event, cannot be resolved at this stage since the human 11β-HSD gene has not yet been cloned. In this regard, the rather delayed peak of 11β-HSD induction (7 days) may suggest a secondary event. However, similar delayed effects of glucocorticoids have been reported (31-33). Our finding that insulin inhibits 11β-HSD induction provides an interesting link between steroid and peptide hormones that regulate cellular metabolism. Half maximal inhibition was observed at physiologic insulin levels (1-2nM). Removal of serum from the incubation medium was necessary to clearly detect this effect. Our results indicate that other negative regulatory factor(s) may be present in serum that utilize cAMP and/or protein kinase C in their second messenger pathways.

The unexpected observation that the Vmax values of the 11oxo-reductase and11β-dehydrogenase activities are modulated in the same direction and to a similar degree by several treatments strongly suggests that, contrary to common belief (19,35-38), a single protein is responsible for both activities. Based on physiochemical properties and clinical data, it was suggested that 11ß-HSD contains two separate enzymes (38). This was supported by the inability to demonstrate reductase activity following purification of 11β-HSD from rat liver microsomes using agarose-NADP affinity chromatography (35). However, recent work, using isoelectric focusing analysis of detergent extracted rat renal 11β-HSD showed a single peak with both 11β-dehydrogenase and 11oxo--reductase activities (39). During preparation of this manuscript cloning and expression of rat liver 11β-HSD was reported and the authors concluded that a single protein catalyzes both activities (40). Differences in redox state therefore likely account for differences in relative activity amongst various tissues (1, 38). Our results in cultured genital skin fibroblasts demonstrate a 11oxo-reductase to 11ß-dehydrogenase rate ratio of 5-10. If indeed a single protein catalyzes both reactions the difference between the Vmax values likely reflects the intracellular NADPH/NADP levels since the Km's for both activities are similar. In experiments with broken cells (data not shown), equal 11ß-dehydrogenase and 11oxo-reductase rates were achieved by adding saturating concentrations of NADP and NADPH, respectively. These data also are consistent with the one enzyme theory.

The augmentation of the 11ß-dehydrogenase rate by the addition of cortisone can best be explained by dilution of the formed [$^3$H] cortisone thereby preventing its rapid conversion back to [$^3$H] cortisol. The observation that not only cortisone but also 11-oxy progesterone increases the 11β-dehydrogenase rate is

compatible with this explanation. Further studies using the release of tritium from cortisol labelled at the 11α position as a more direct measure of 11β-dehydrogenase activity are needed to substantiate this conclusion. The apparent enhancement of the enzyme activity by addition of product may be important in the interpretation of other studies of 11ß-HSD. For example, failure to consider this problem may have contributed to the conclusion that some cells contain 11oxo-reductase without 11β-dehydrogenase or vice versa (36).

11β-HSD has been implicated in conveying specificity of aldosterone receptors to aldosterone (4-7) and in protecting non-glucocorticoid target tissues from the deleterious effects of cortisol (41). We propose a more global function of this enzyme. By virtue of having both 11ß-dehydrogenase and 11oxo-reductase activities that are subject to metabolic control the enzyme can modulate the effectiveness of circulating cortisol and/or cortisone differently in many glucocorticoid target cells. We have shown that cortisone induces aromatase activity and even 11β-HSD itself in cultured skin fibroblasts. Moreover, inducing 11β-HSD by cortisol or serum removal, increased the apparent effectiveness of cortisone by accelerating its conversion to cortisol. That cortisone can be activated in vitro is not suprising since it was the first glucocorticoid to be used in clinical medicine. Recent studies have shown that cortisone is effective in two other cell culture systems (28, 42). Since 11β–HSD can effectively modulate the availability of intracellular cortisol to glucocorticoid receptors, it may play a role in the phenomenon of cortisol resistance described in humans (42, 43), new world primates (44) and the guinea pig (45), or in cortisol hypersensitivity in humans (46). Experiments to be described elsewhere have shown that 11β-HSD activity in skin fibroblasts from the squirrel monkey is 50-100 fold higher than that found

in human cells and interestingly, the relative activities favor formation of cortisone rather than cortisol[2].

**REFERENCES**


1) Murphy, B.E.P. (1981) J. Steroid Biochem. **14**, 811-817.

2) Weindenfeld, J., Siegel, R.A., Levy, J., and Chowers, I. (1982) J. Steroid Biochem. **17**, 357-360.

3) Funder, J.W. (1987) Science **237**, 236-237.

4) Edwards, C.R.W., Burt, D., and Stewart, P.M. (1989) J. Steroid Biochem. **32**, (1B), 213-216.

5) Edwards, C.R.W., Stewart, P.M., Burt, D., Brett, L., McIntrye, M.A., Sutanto, W.S., DeKloet, E.R., and Monder, C. Lancet, (October 29, 1988) 986-989.

6) Funder, J.W. (1989) Endorcrine Research **15** (1 and 2), 227-238.

7) Funder,J.W., Pearce, P.T., Smith, R., and Smith, A.I. (1988) Science **242**, 583-585.

8) Arriza, J.L., Weinberger, C., Cereili, G., Glaser, T.M., Hendelin, B.L. Housman, D.E., and Evans, R.M. (1987) Science **237**, 268-275.

9) Sheppard, K.E., and Funder, J.W. (1987) J. Steroid Biochem. **28**(6), 737-742.

10) Spieth, A., Hierholzer, K., Techn. Asst. Lichtenstein, L., and Siebe, H. (1987) Pfluger Arch. **480** R42.

11) Rossier, B.C. (1989) Endocrine Research **15**(1 and 2), 203-226.

12) Doerr, H.G., Sippell, W.G., Versmold, H.T., Bidlingmaier, F., and Knorr, D. (1988) Pediatric Research **23**(5), 525-529.

13) Wei J.Q., Zhou, X.T., and Wei, J.L. (1987) Clin. Chem. **33**(8), 1354-1359.

14) Meulenberg, P.M.M., Ross, H.A., Swinkels, L.M.J.W., and Bernard, T.J. (1987) Clinica Chemica Acta **165**, 379-385.

15) Bradford, M.M. (1976) Analytical Biochem. **72**, 248-254.

16) Thompson, E.A. Jr., and Siiteri, P.K. (1974) J. Biol. Chem. **249**, 5364-5372.

17) Lopez-Bernal, A., Flint, A.P.F., Anderson, A.B.M., and Turnbull, A.C. (1980) J. Steroid Biochem. **13** 1081-1087.

18) Murphy, B.E.P., Vedady, D. (1981) J. Steroid Biochem. **14**, 807-809.

19) Lakshmi, V., and Monder, C (1985). Endocrinology **116**(2), 552-560.

20) Kobayashi, N., Schulz, W., and Hierholzer, K. (1987) Pfluger Arch. **408** 46-53.

21) Mahesh, V.B., and Ulrich, F. (1960) J. Biol. Chem. **235**(2), 356-360.

22) Berliner, DL., and Ruhmann, A.G. (1966) Endocrinology **78**, 373-382.

23) Berliner, D.L., and Dougherty, T.F. (1961) Pharm. Rev. **13**, 329-359.

24) Zumoff, B., Bradlow, H.L., and Levin, J (1983) J. Steroid Biochem. **18**(4) 437-440.

25) Koerner, D.R., and Hellman, L. (1964) Endocrinology **75**, 592-601.

26) Pepe, G.J., Waddell, B.J., Stahl, S.J., and Albrecht, E.D. (1988) Endocrinology **122** 78-83.

27) Abramovitz, M., Carriero, R., and Murphy, B.E.P. (1984) J. Steroid Biochem. **21**(6) 677-683.

28) Smith, B.T., Torday, J.S., and Giround, C.J.P. (1973) Steroids **22** 515-524.

29) Dougherty, T.F., Berliner, M.L., and Berliner, D.L. (1960) Endocrinology **66**, 550-558.

30) Tye, L.M., and Burton, A.F. (1980) Life Sciences **26**, 35-39.

31) Brinberg, N.C., Lissitzky, J.C., Hinman, M., and Herbert, E. (1983) Proc. Nat'l. Acad. Sci. USA **80**, 6982-6986.

32) Paek, I., and Axel, R. (1987) Molecular and Cellular Biology 7(4), 1496-1507.

33) Johnson G.S., and Jaworski, C.J. (1983) Molecular Pharmacology, **23**, 648-652.

34) Lakshmi, V., and Monder, C. (1988) Endocrinology **123** 2390-2398.

35) Abramavitz, M., Branch, C.L., and Murphy, B.E.P. (1982) J. Clin. Endocrinol. Metab., **54** 563-568.

36) Stewart, P.M., Corrie, J.E.T., Shackleton, C.H.L., and Edwards, C.R.W. (1988) J. Clin. Invest. **82**, 340-349.

37) Monder, C., and Shackleton, C.H.L. (1984) Steroids **44**(5), 383-417.

38) Schulz, W., Lichtenstein, I., Siebe, H., and Hierholzer, K. (1989) J. Steroid Biochem. **32**(4), 581-590.

39) Agarwal, A.K., Monder, C., Eckstein, B., and White, P.C. (1989) J. Biol. Chemistry **264**(32), 18939-18943.

40) Phillips, D.M., Lakshmi, V., and Monder, C. (1989) Endocrinology **125**, 209-216.

41) Siminoski, D., Murphy, R.A , Rennert, P., and Heinrich, G. (1987) Endocrinology **121**, 1432-1437.

42) Chrousos, G.P., Loriaux, D.L., Brandon, D., Tomia, M., Vingerholds, A.C.M., Merriam, G.R., Johnson E.O., and Lipsett, M.B. (1983) J. Steroid Biochem **19**(1), 567-575.

43. Iida, S., Gomi, M., Moriwaki, K., Itoh, Y., Hirobe, K., Matsuzawa, Y., Katagiri, S., Yonezawa, T., and Tarui, S.C. (1985) J. Clin. Endocrinol. Metab. **60**,967-971.

44. Chrousos, G.P., Renquist, D., Brandon, D., Eil, C., Pugeat, M., Vigersky, R., Cutler, G.B. J.r., Loriaux, D.L., and Lipsett., M. B. (1982) Proc. Natl. Acad. Sci. USA. **79**, 2036-2040.

45. Kraft, N., Hodgson, A.J., and Funder, J.W. (1979) Endocrinology **104**:344-349.

46. Iida, S., Nakamura, Y., Fujii, H., Nishimura, J.I., Tsugawa, M., Gomi, M., Fukata, J., Tarui, S., Moriwaki, K., and Kitani, T. (1990) J. Clin. Endocrinol. Metab. **70**:, 729-737.

* This work was supported by NIH grant CA-27702 (to P.K.S.)

+ To whom correspondence should be addressed.

Reproductive Endocrinology Center

University of California San Francisco

San Francisco, California, 94143

Phone: (415) 476-2719

Fax: (415) 476-753-3271

1 The abbreviations used are: 11ß-HSD, 11ß-hydroxysteroid dehydrogenase; DEX, dexamethasone; DME, Dulbecco's Modified Eagle's medium; FGF, fibroblast grwoth factor, EGF, epidermol growth factor, cAMP, 8-bromo cyclic AMP; CS, Calf serum; TLC, thin layer chromntography; DCC, dextran-coated charcoal, SCS, chorcoal stripped calf serum; PMA, phorbol myristate acetate.

2 11ß-Hydroxysteroid dehydrogenase and glucocorticoid resistance in new world primates. Muhammed M. Hammami and Pentti K. Siiteri (1990). Submitted.

Fig. (1)

Basal 11oxo-reductase and 11ß-dehydrogenase activities in human genital skin fibroblasts. (a) 11Oxo-reductase time course using 995nM [$^3$H] cortisone (b) 11Oxo-reductase saturation plot at 10 minutes. (c) 11ß-Dehydrogenase time course using 300nM [$^3$H] cortisol alone (control) or with 100-fold excess cortisone. (d) 11ß-Dehydrogenase saturation curve at 1 hr in the presence of 100 fold excess unlabeled cortisone. Results are the mean of duplicate assays, and are typical of three experiments (± deviation from the mean).

Fig. (2)

Specificity of glucocorticoid induction of 11β-HSD. (a) 11Oxo-reductase activity measured after 72 hrs incubation with the indicated hormones. (b) 11ß-Dehydrogenase activity measured after 60 hrs incubation with the indicated hormones. Dex, dexamethasone 100nM; $E_2$, estradiol 50nM; prog, progesterone 5µm; $T_3$, triiodothyronine 2nM; DHT, dihydrotestosterone 50nM; T, testosterone 500nM. Both experiments were done in the presence of 10% SCS. Control reductase and dehydrogenase activities were 0.36 and 2.8 pmol/mg protein/h respectively using 30nM substrate. Bars represent the mean of duplicate assays (± deviation from the mean). Similar results were obtained when cells were incubated, with hormones for 18 hrs.

Fig. (3)

Dexamethasone induction of 11oxo-reductase activity. (a) Time course. Cells were incubated with 100nM dexamethasone in 10% SCS for the indicated

times. (b) Dose response curve. Cells were incubated for 60 hrs with the indicated concentrations of dexamethasone in 10% SCS. [$^3$H] cortisone concentration in the assay was 55nM in (a) and 20nM in (b). Bars represent the mean of duplicate assays. (± deviation from the mean). Results are typical of two experiments.

-

Fig. (4)

Inhibitory effect of serum on 11oxo-reductase activity. (a) serum withdrawal time course. (typical of two experiments). Cells were incubated in media containing 4mg/ml human albumin for the indicated times or maintained in 10% SCS as control. 11oxo-reductase activity in the control was 2pmol/mg/h using 55nM [$^3$H] cortisone. (b) serum dose response (typical of two experiments). Cells were incubated in media containing 4mg/ml human albumin (SF) with or without human serum at the indicated concentrations for 72h. 11Oxo-reductase activity of control cells was 1.46 pmol/mg protein/h using 15nM [$^3$H]-cortisone. Bars represent the mean of duplicate assays (± deviation from the mean).

Fig. (5)

Insulin inhibition of 11β-HSD activity. Cells were incubated with 10% SCS (SCS) or 4mg/ml human albumin with or without the indicated doses of insulin for 5 days. Media was changed every 48 hours. 11Oxo-reductase activity was determined using 33nM [$^3$H] cortisone. Bars represents the mean of duplicate assays (± deviation from the mean). Results are typical of three experiments.

Fig. (6)

Aromatase induction by cortisol or cortisone. Cells in 10% SCS were incubated with or without 200nM cortisol or 200nM cortisone for the indicated times. Aromatase activity was determined as described in methods. Bars represent

the mean of duplicate assays (± deviation from the mean). Results are typical of two experiments.

## TABLE 1

Cortisol induction of fibroblast 11oxo-reductase activity in the presence or absence of glucocorticoid receptor antagonists.[a]

| | 11Oxo-reductase (pmol/mg protein/h) | | |
|---|---|---|---|
| CORTISOL(μM) | CONTROL | RU486 | DM |
| 0 | 1.4 | - | - |
| .02 | 1.5 | 1.4 | 2 |
| . 2 | 2.6 | 1.5 | 1.9 |
| 2 | 3.4 | 2.0 | 2.4 |
| 20 | 4.1 | 3.3 | 3.6 |

[a]Cells were treated with cortisol for 90 hrs in 10% SCS prior to washing and addition of substrate (20nM). Values are the mean of duplicate assays and results are typical of two experiments. DM is dexamethasone mesylate (2 μM).

# TABLE 2

Modulation of 11β-HSD activity by serum, glucocorticoids and insulin.

a) 11Oxo-reductase (pmol/mg protein/h)

| | Insulin | |
|---|---|---|
| | - | + |
| Control | 4.9 | - |
| Cortisol (20μm) | 15 | 6.4 |

b) 11β-Dehydrogenase

| | - | + |
|---|---|---|
| Control | 0.5 | 0.2 |
| SF | 1.2 | 0.6 |
| Dex(100nM) | 1 | 0.2 |

c)

| | 11Oxo-reductase | | 11β-Dehydrogenase | |
|---|---|---|---|---|
| | 10% SCS | SF | 10% SCS | SF |
| No steroids | 1.7 | 4.6 | 0.34 | 0.9 |
| Cortisone (20μM) | 4.9 | 11.9 | 0.5 | 1.4 |
| Cortisol (20μM) | 2.4 | 8.7 | 0.36 | 1.1 |

**TABLE 2** (cont.)

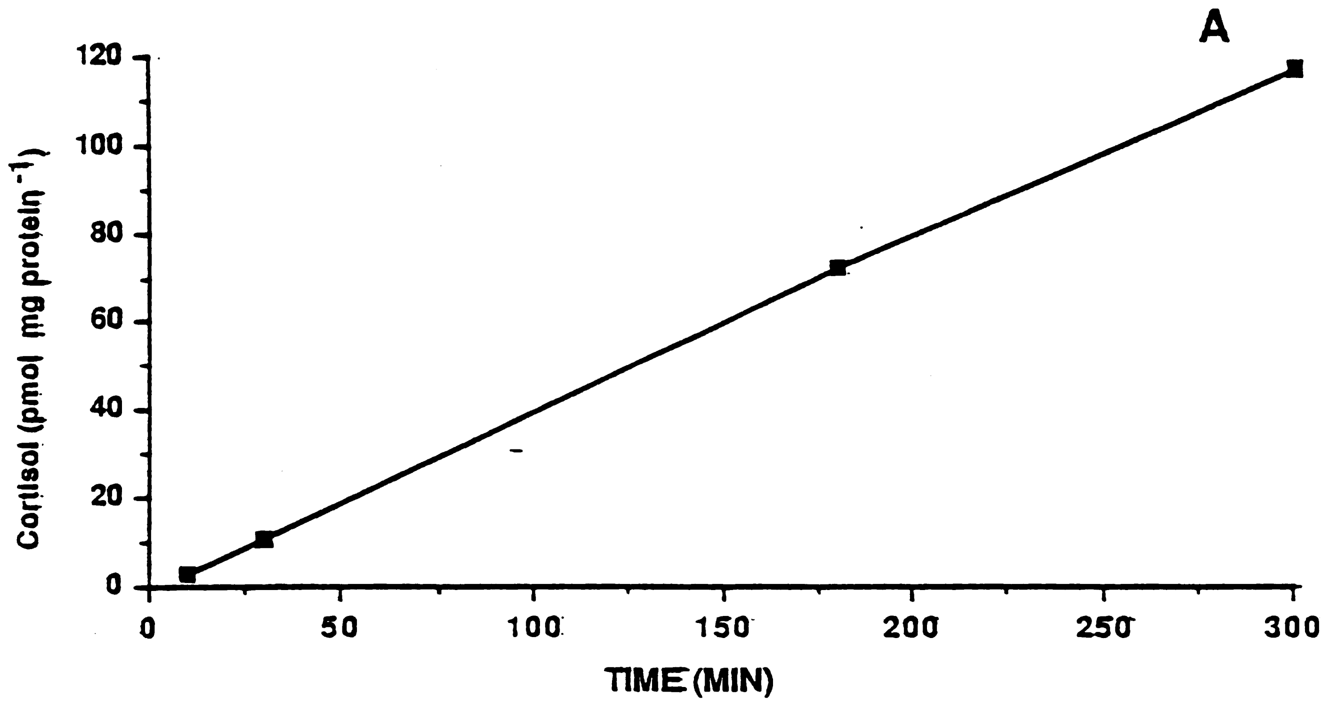
a) Insulin prevents cortisol induction of 11oxo-reductase. Cells were incubated for 5 days in 10% SCS alone (control) or with 20μM cortisol, with or without 10μM insulin. b) Insulin blocks 11β-dehydrogenase induction by Dex or serum withdrawal (typical of three experiments). Cells were incubated for 5 days in 10% SCS, SF or with 100nm Dex in 10% SCS, all with or without 10μM insulin. c) Induction by cortisol (or cortisone) and by serum withdrawal is additive for both 11β-reductase and 11β-oxidase (typical of two experiments). Cells were incubated under the indicated conditions for 70 hrs. Values in (a-c) are the mean of duplicate assays and are in pmol/mg protein/h. Substrate concentrations were 30nM in a and b and 25nM in c.

## TABLE 3

Kinetic studies of basal and induced 11oxo-reductase and 11β-dehydrogenase activities.[a]

| | 11Oxo-reductase[b] | | 11β-Dehydrogenase[b] | |
|---|---|---|---|---|
| | Km[c] | Vmax[c] | Km[c] | Vmax[c] |
| Basal[d] | 219; 298 | 20; 28 | 220; 229 | 4; 1.9 |
| Dex[d] | 230; 204 | 62; 61 | 233; 293 | 13.7; 7.6 |
| SF[d] | 230; 229 | 120;141 | 312;470 | 14.5; 8.9 |

[a]Induction was for 120 hrs with 100 nM Dex in 10% SCS (Dex) or with medium containing 4mg/ml human albumin (SF). Controls were incubated with 10% SCS (basal). [b]Each analysis is the result of 12 determinations of enzyme activity at substrate concentrations ranging from 24 to 995 nM. Standard errors were less than 20%. [c]Km is in nM, Vmax is in pmol/mg protein/h. [d]Two seperate experiments.



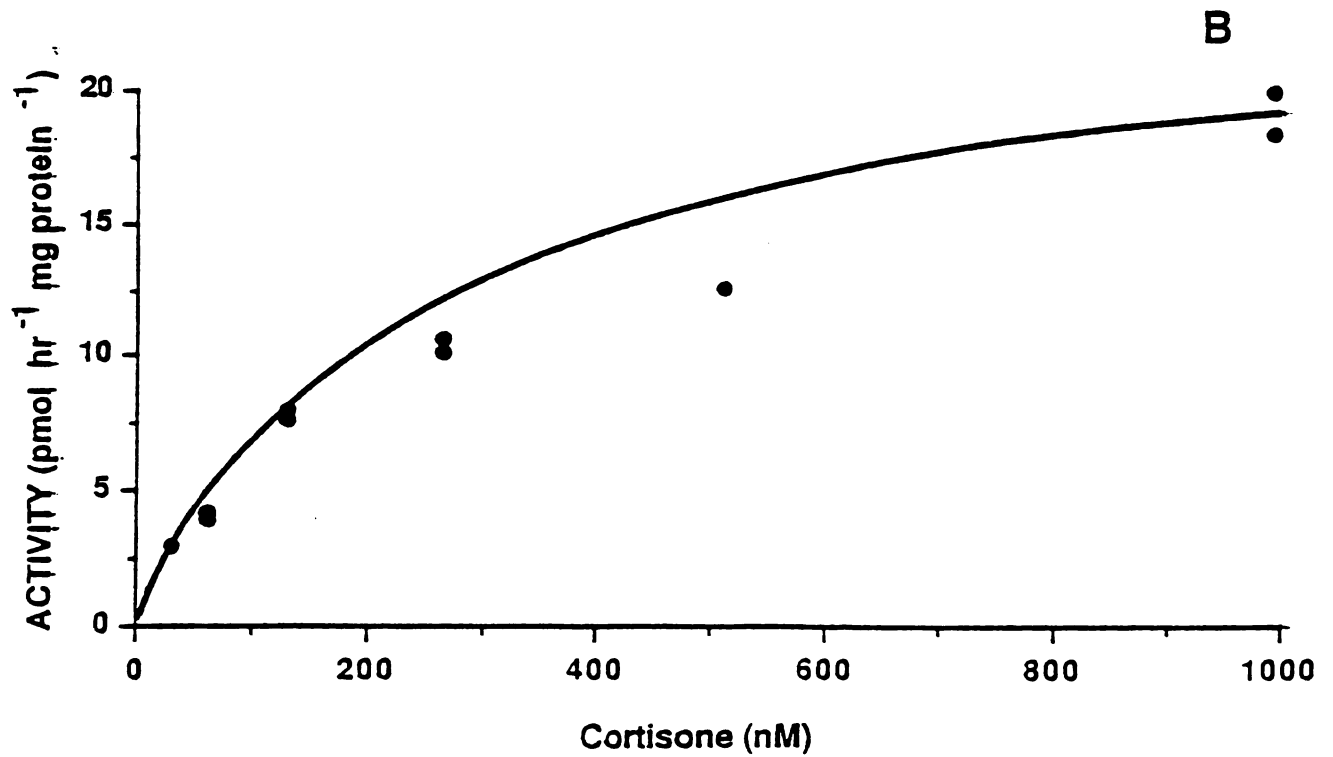
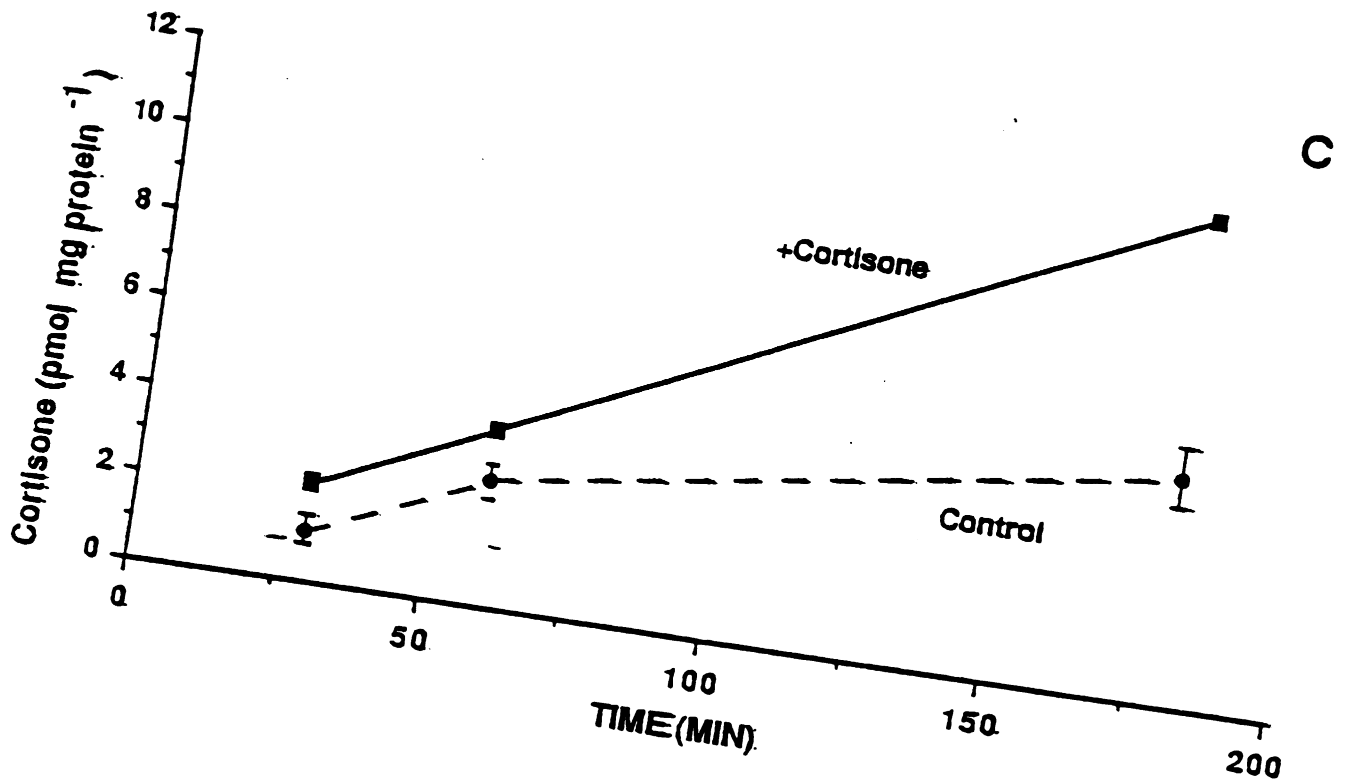




Fig-1



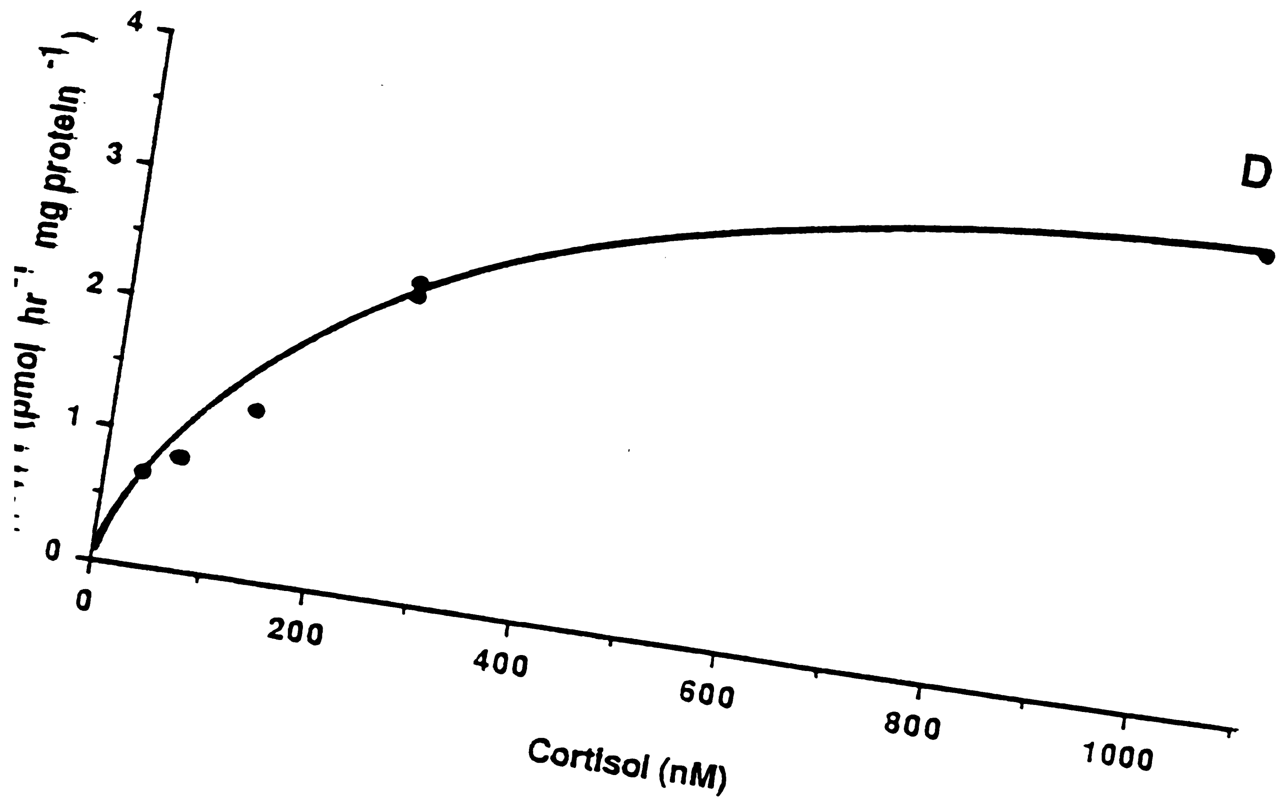
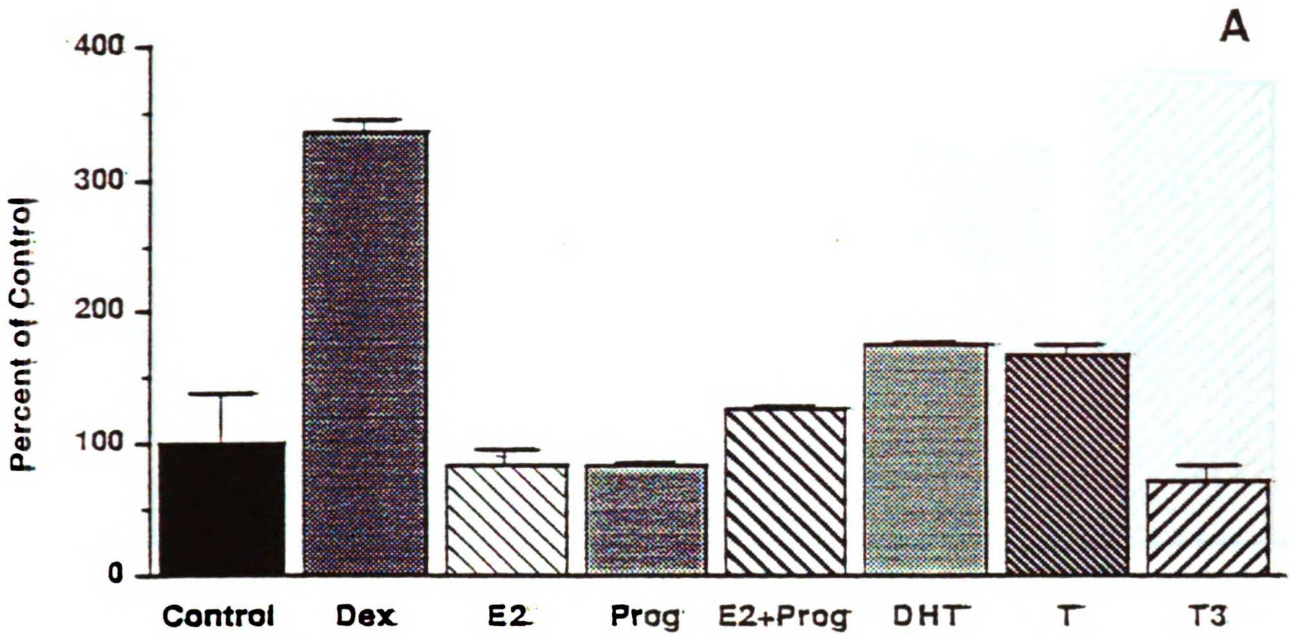




Fig - 1



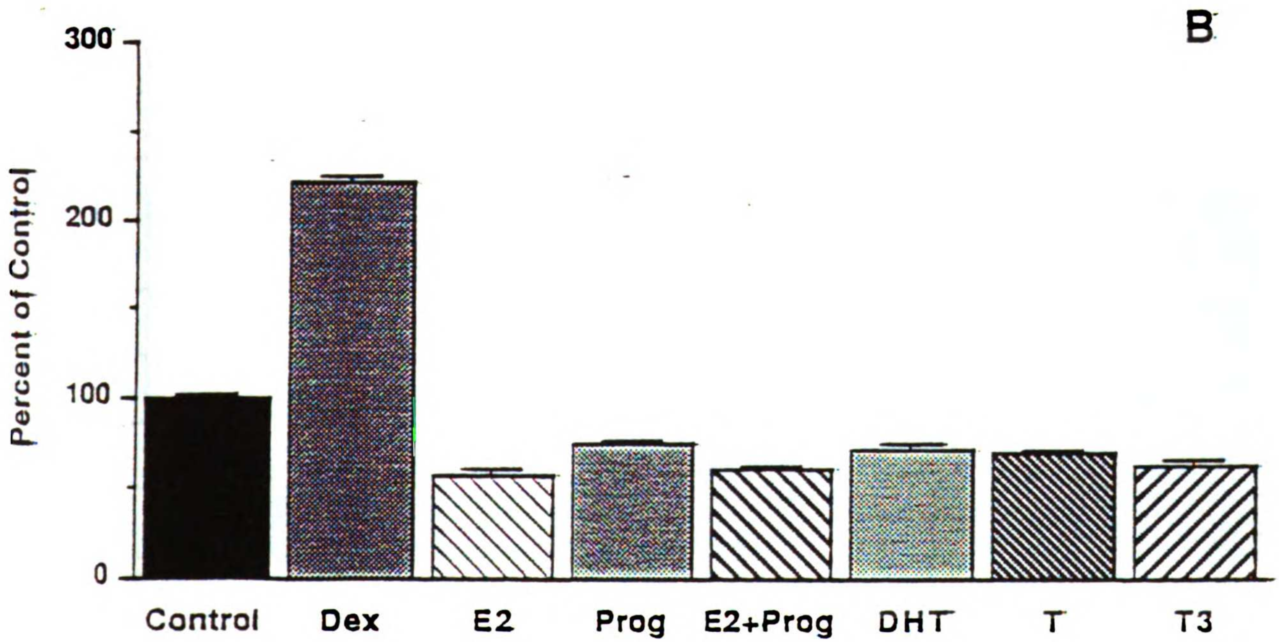
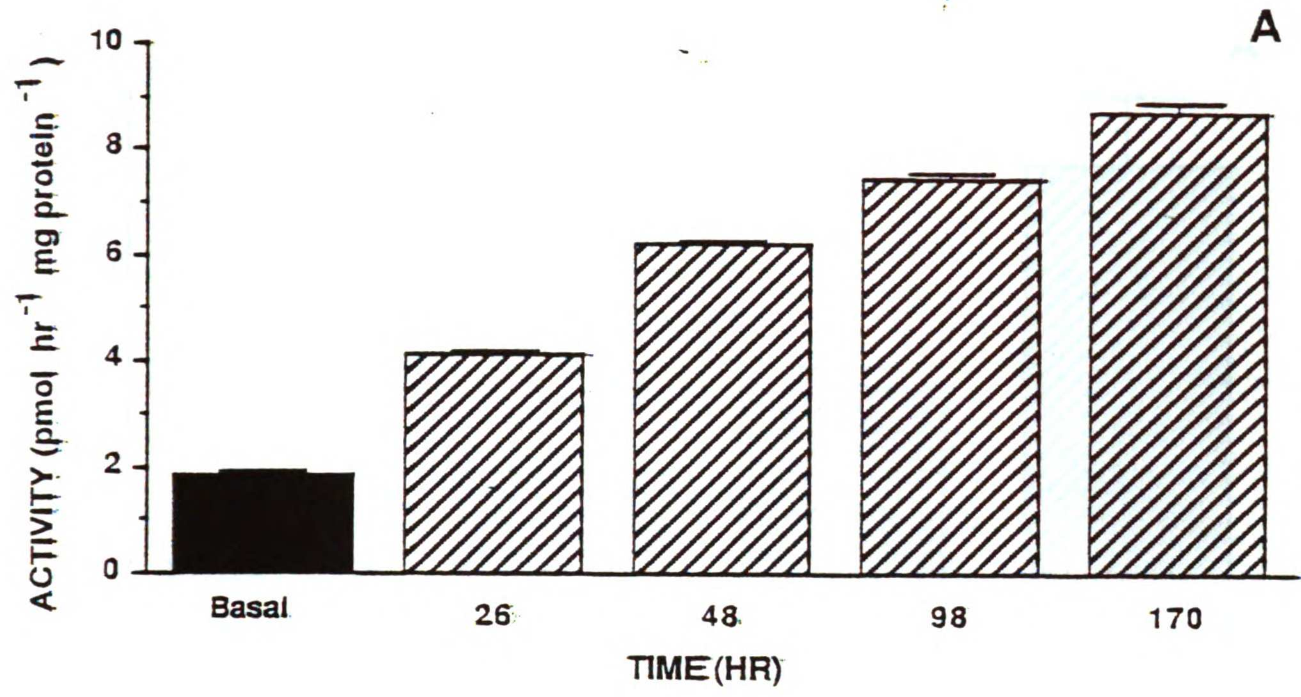




Fig-2



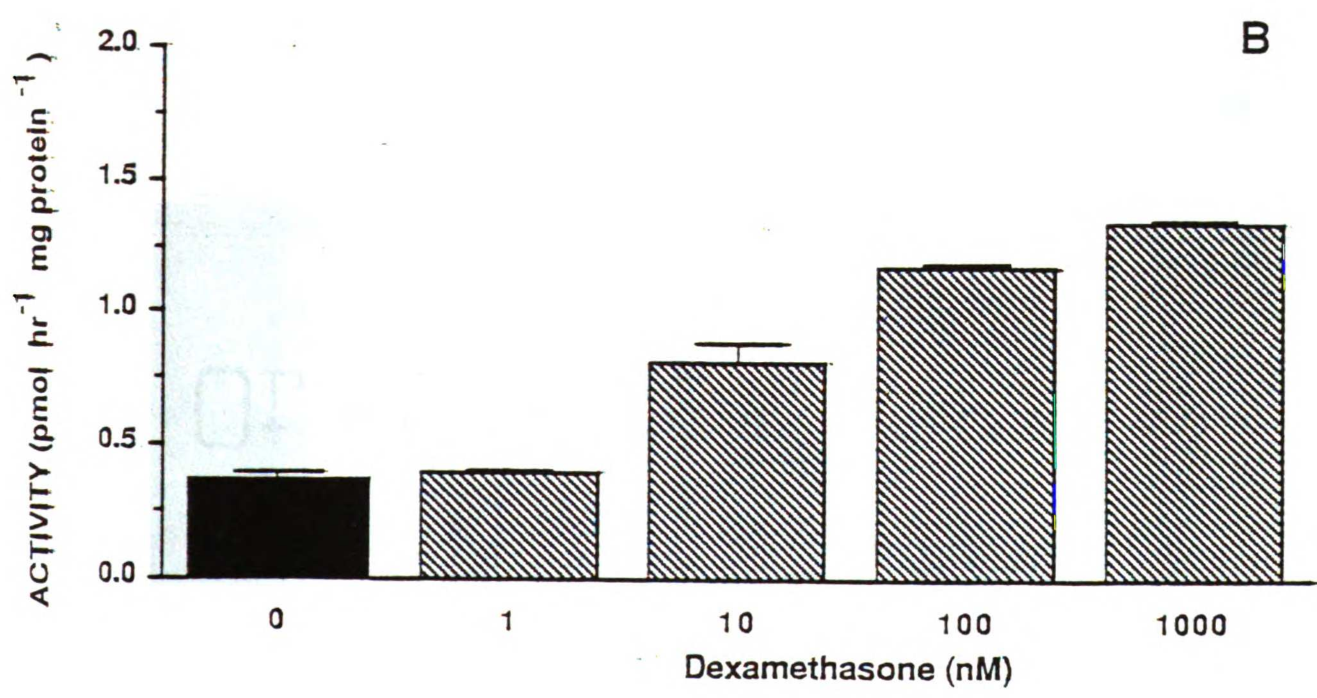
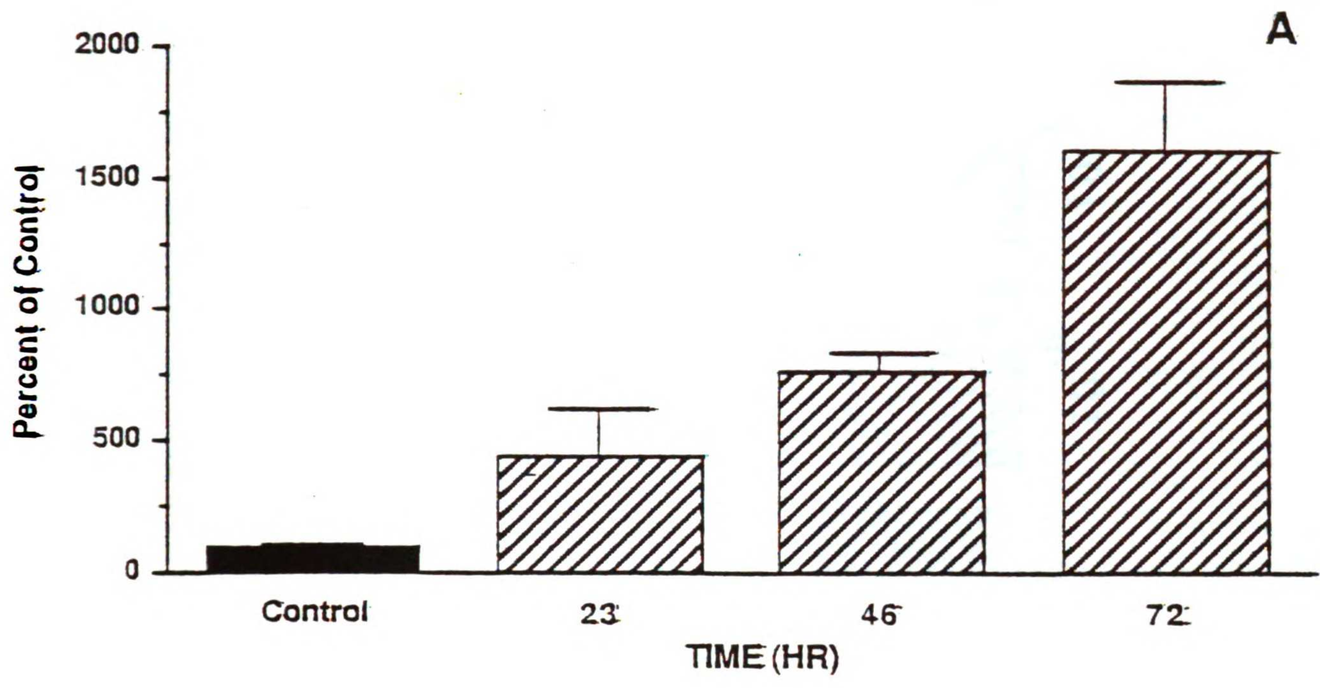




Fig 3

51d



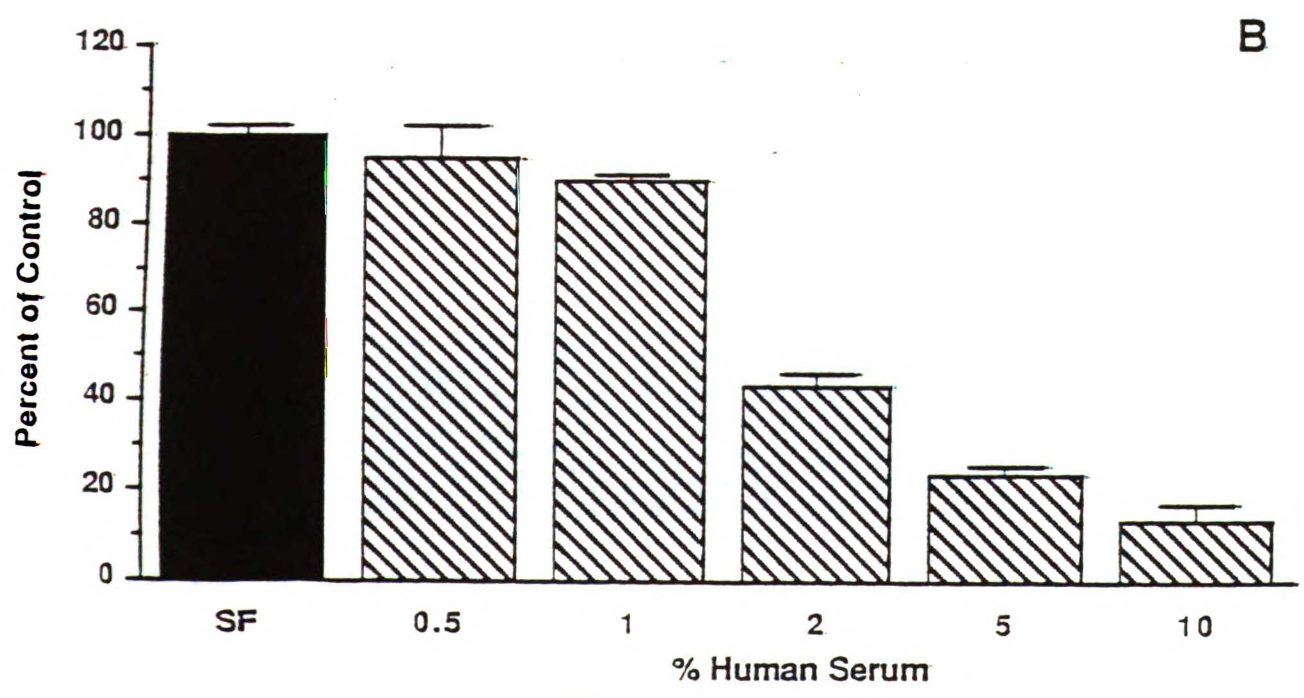




Fig 4

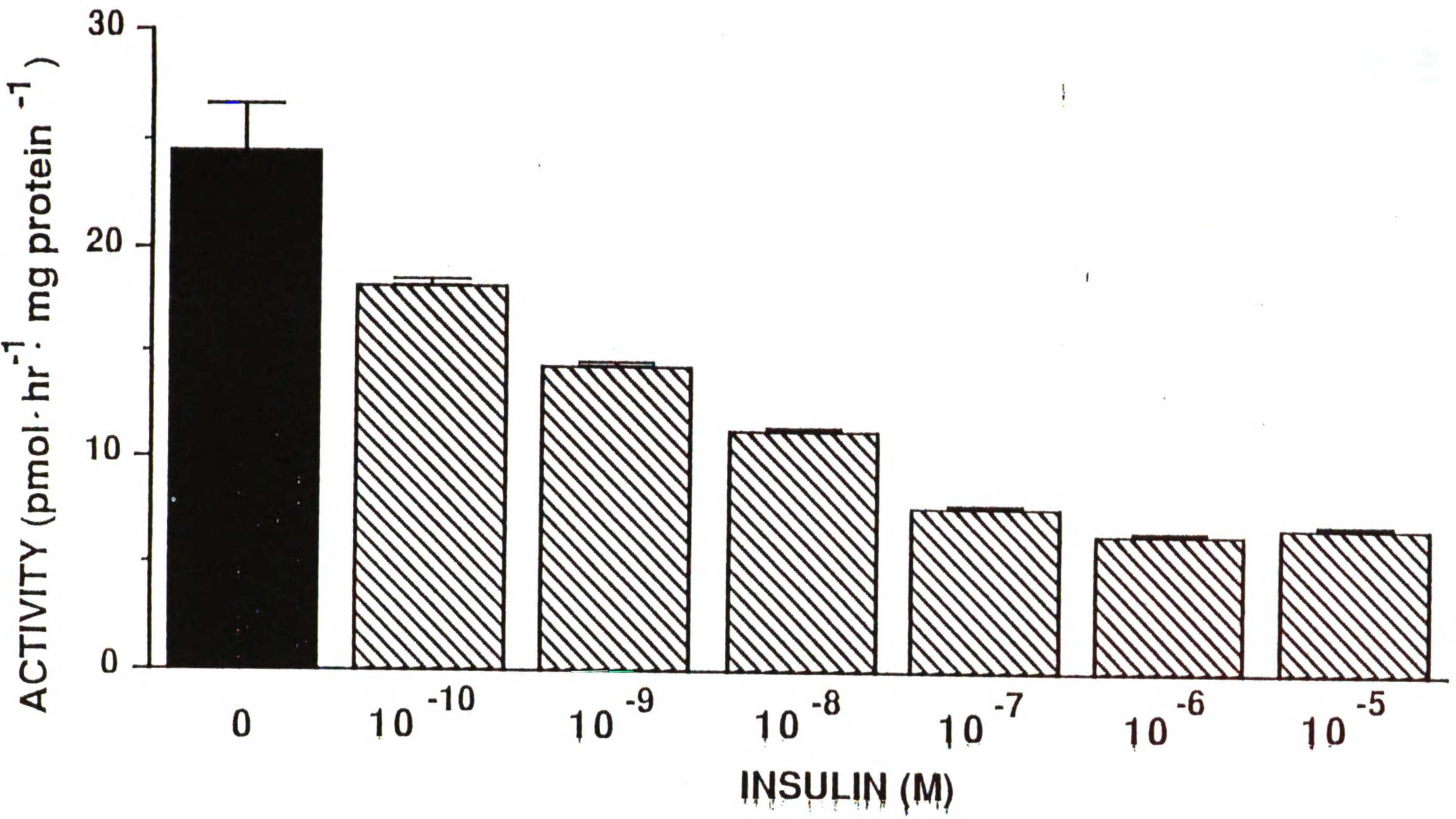



Fig 5

# 11ß-HYDROXY STEROID DEHYDROGENASE (11ß-HSD) AND GLUCOCORTICOID RESISTANCE IN NEW WORLD PRIMATES *

## (REGULATION / SQUIRREL MONKEY / NADPH-NADP RATIO)

Muhammad M. Hammami and Pentti K. Siiteri†

Reproductive Endocrinology Center

University of California San Francisco

San Francisco, California 94143

Classification: Medical Physiology, Endocrinology and Metabolism

## ABSTRACT

Glucocorticoid resistance in South American primates is thought to be due to defective glucocorticoid receptors. We have investigated the possible role of 11ß-HSD, a pyridine nucleotide dependent enzyme that interconverts cortisol and cortisone, in this phenomenon by comparing 11ß-HSD activities in squirrel monkey (Sm), rhesus monkey and human skin fibroblast cell lines. Using 40nM cortisol as substrate, 11ß-dehydrogenase activity was similar in human and rhesus monkey cells but at least 25 fold higher in Sm cells. Kinetic studies indicated that 11ß-dehydrogenase has a 500 fold higher Vmax whereas the Vmax for 11oxo-reductase activity was only 70-100 fold higher in Sm cells than in human cells. Therefore, the ratio of 11Oxo-reductase to 11ß-dehydrogenase activities in Sm cells was about one rather than 5-10. This shift in favor of cortisone formation appears to be due to a lower redox state in Sm cells since the relative 11ß-dehydrogenase activity could be further increased by removing glucose from the culture medium or by incubating Sm cells at $4^{o}$ instead of $37^{o}$. In human cells, 11ß-HSD activity is markedly increased by dexamethasone treatment or serum removal and decreased by insulin, whereas, these treatments had no effect in Sm cells. These results indicate that 1) 11ß-HSD is consitutively overexpressed in Sm cells, and 2) that the enzyme favors conversion of active cortisol to inactive cortisone due to differences in intermediary metabolism of glucose. High 11ß-HSD activity in glucocorticoid target cells may play a primary role in some forms of glucocorticoid resistance by lowering the extremely high extracellular cortisol to levels that are appropriate for receptor binding. Under normal circumstances 11ß-HSD may provide the long sought link between the hypothalamic-pituitary-adrenal axis and food intake.

## INTRODUCTION

Generalized cortisol resistance is rare in humans (1, 2), but commonplace in new world primates (3). Stigmata of Cushing's syndrome are not present despite plasma cortisol levels that are 20-30 fold higher in the squirrel monkey (Sm) as compared to normal humans. A decrease in the affinity and/or binding capacity of otherwise normal glucocorticoid receptors (GR) was postulated to be the primary cause of cortisol resistance in new world primates (3, 4). However, the difference in GR affinity for synthetic glucocorticoids in skin fibroblasts from old and new world primates was only about 2 fold (3). Moreover, although the binding affinity of aldosterone to aldosterone receptors (AR) in the squirrel monkey was similar to that found in humans, the ability of cortisol to compete for aldosterone binding was 27 fold lower (5), suggesting a more generalized abnormality related to cortisol itself rather than to GR or AR.

Previously we have suggested that altered metabolism of steroids may play a role in the resistance to multiple steroid hormones in new world primates (6). In the present study we have investigated the possible involvement of 11ß-HSD in cortisol resistance in the squirrel monkey. We found that 11ß-HSD is highly overexpressed in cells from squirrel monkeys, as compared to those from an old world primate or humans. Moreover, both 11ß dehydrogenase and 11oxo-reductase activities were found in all cells but they are shifted in favor of cortisone production in squirrel monkey cells. The results suggest that 11ß-HSD may play a primary role in cortisol resistance in this species by diverting cortisol from interaction with glucocorticoid receptors by conversion to inactive cortisone.

# MATERIALS AND METHODS

## MATERIALS

Dulbecco's Modified Eagle's medium (DME) (4.5g/L glucose, 0.584g/l L-Glutamine, 3.7g/L NaHC03) free of phenol; DME without glucose; fungizone 250mg/ml; penicillin G 10,000 units/ml and streptomycin sulfate 10,000 mcg/ml; insulin 1mg/ml (24u/mg); and calf serum were obtained from the tissue culture facility of University of California, San Francisco, Six well plates were obtained from Becton Dickinson Labware, Lincoln Park, New Jersey. [1,2, $^{3}$H]-labeled cortisone and [1,2,6,7, $^{3}$H]-labeled cortisol were obtained from Amersham, Arlington Heights, Illinois and New England Nuclear, Boston, MA respectively; nonradioactive steroids from Steraloids Inc., Wilton, NH; and human albumin (essentially fatty acid and globulin free) from Sigma, St. Louis, MO. The following cell lines were obtained from the tissue culture facility of University of California, San Francisco, CA: Sm P2F, Sm 1-F, Sm B7-F (squirrel monkey male genital skin fibroblasts); Sm B1-La, Sm 5.La (squirrel monkey labial skin fibroblasts); Sm KSK (male, peripheral skin fibroblasts); Sm fat-02 and Sm fat-04 (omental stromal fat from male and female animals respectively); Rhm K-F3, Rhm 2-F (male rhesus monkey genital skin fibroblasts); Rhm La-1 (labial skin fibroblasts); 560D, HS5555K, HS681, 26D, HS547 (normal human, male genital skin fibroblasts); 628D (human genotipic female with clitoromegaly). Thin layer chromatography plates coated with silica gel were obtained from Whatman Ltd. Kent, England.

## METHODS

Cells were maintained in DME (phenol free) medium supplemented with 10% calf serum (CS), penicillin (100 units/ml) and streptomycin (100mg/ml), amphotericin b (Fungizone; 2-5mg/ml) and fibroblast growth factor (2ng/ml). Cells were plated in 6-well plates and fed with medium and grown until confluent or near confluent. After washing three times with DME, cells were incubated with substrates or effectors for the indicated times with or without additions.

Tritium labelled cortisol and cortisone (specific activities of 99.8 and 29ci/mmol, respectively) were purified by celite column chromatography prior to use. The solvent systems were iso-octane, t-butanol, $H_20$ (250; 125; 225) for cortisol and (100: 35: 90) for cortisone. The eluted peaks were collected, dried and reconstituted in ethanol and kept at $4^oC$. After purification, [$^3$H]-cortisone contained less than 1% cortisol and [$^3$H]-cortisol less than 2% cortisone as determined by thin layer chromatography (TLC). The data points were corrected for the amount of cross contamination as determined in each experiment.

Enzyme assays with intact cells: monolayers of cells in 6-well plates were incubated with [$^3$H]-cortisol (dehydrogenase) or [$^3$H]-cortisone (reductase) in DME at $37^oC$ (CO2 5%, air 95%) unless stated otherwise, for the indicated times. An aliquot of the medium was then extracted with 10 volumes of ethyl acetate. The ethyl acetate extract was evaporated under a stream of nitrogen and the radioactivity was reconstituted with 100ml ethyl acetate containing cortisol and cortisone standards (1mg/ml). Duplicate aliquots were applied to TLC plates and another aliquot was counted to monitor recovery. TLC plates were developed in chloroform: ethanol: t-butanol (9:1:1), dried and the standard spots visualized by

first spraying with a mixture of glacial acetic acid, $H_2SO_4$(conc.) and p-anisaldehyde (50:1:1) and then heating in a vacuum oven at 100°C for 5-10 minutes. The identified cortisol and cortisone spots were cut out, eluted with 0.3ml of ethanol into mini-vials and the tritium counted after adding scintillation fluid. In preliminary experiments it was found that the cortisol and cortisone spots accounted for more than 95% of the total radioactivity recovered from the plates. Negligible radioactivity was associated with 20α or 20ß-reduced cortisone standards both of which were clearly separated from the cortisol standard. The recovery of total radioactivity applied to the TLC plates averaged 80% and did not vary significantly between the controls (no cells) and the experimental points. Cell layers, washed twice with DME, were solubilized by incubating overnight with 0.5N NaOH at room temperature and cellular protein (about 300 µg per well) was determined by the Bradford assay (7). Results are expressed as pmol/mg protein/hour of the product formed and were calculated by multiplying the percentage of substrate converted by the initial amount of substrate and then adjusting for protein and time of incubation. For kinetic studies, the data were linearized according to Eadie-Scatchard analysis (V/S=Vmax/Km-V/Km, where V is the rate in pmol/mg/h and S is the substrate concentration in nM). A least squares best fit computer program was used to calculate the km and Vmax values.

Controversy currently exists as to whether 11ß-dehydrogenase and 11oxo reductase activities of 11ß-HSD are due to one or two separate enzymes (8,9). Although we have not addressed this issue directly, the results from this and two other studies using fibroblasts from three different species are entirely consistent with a single protein having both catalytic properties. In either case, the results of the present experiments using whole cells and the conclusions drawn are not influenced by this issue.

Stock solutions of steroids and drugs prepared in ethanol, and stored at 4°, were evaporated under a stream of nitrogen and reconstituted in DME before being added to cells. Calf serum used to implement culture medium was treated twice with dextran coated charcoal for 1 1/2 hours at 37° to remove endogenous steroids.

## RESULTS

11ß-HSD activity in squirrel monkey (Sm), rhesus monkey (Rh) and human (Hu) cell lines: Squirrel monkey cell lines obtained from eight different animals were screened for 11oxo-reductase and 11ß-dehydrogenase activities. The cell lines were from different subspecies (Bolivian and Peruvian), different sexes (6 males, 3 females) and different organs (foreskin, labia, peripheral skin and omental stromal fat). Table 1 shows 11oxo-reductase and 11ß-dehydrogenase activities measured at 30 minutes, using 40nM [$^3$H]-cortisone or [$^3$H]-cortisol, respectively, as substrates. Under these assay conditions (37°, 4.5g/L glucose) 11oxo-reductase activity ranged from 35 (omentum stromal fat) to 160 (foreskin) with a mean of 92 pmol/mg protein/h. 11ß-Dehydrogenase activity averaged 18 pmol/mg protein/h. There was no apparent difference between sexes or subspecies. The time of the assay was chosen so that measurable activity could be detected in both human and rhesus monkey cell lines. However, as will be shown later, this tends to underestimate the 11ß-dehydrogenase rate in Sm cells. Table 1 also shows both 11ß-HSD activities in rhesus monkey cell lines (two foreskin, one labial) and six human cell lines (five foreskin, one labial). The human labial cell line is from a female patient with clitoromegaly. All other cell lines were from normal subjects. 11Oxo-reductase activity averaged 8 and 4 pmol/mg protein/h in the Rh and Hu cell

lines respectively, whereas the average 11ß-dehydrogenase activity in both species was 0.7 pmol/mg protein/h.

<u>Characterization of 11oxo-reductase and 11ß-dehydrogenase activities in Sm cells</u>: One Sm cell line (Sm-5La) was selected for further study. Fig. 1 (a) and (b) depicts 11oxo-reductase and 11ß-dehydrogenase activities respectively, measured at 10 minutes, using several substrate concentrations. The data from similar experiments performed on two different passages (P5 and P25) were transformed according to Eadie-Scatchard analysis. Table 2 (a) and (b) summarizes these results. At 10 minutes, the apparent Vmax was 584±124 and 1653±325 pmol/mg protein/h for 11ß-dehydrogenase and 11oxo-reductase respectively. The Km of both activities was about 400 nM. We then examined the time course of conversion of [$^3$H]-cortisol to [$^3$H]-cortisone and of [$^3$H]-cortisone to [$^3$H]-cortisol with or without unlabeled products. The results are shown in Fig. 2. It is clear that while the conversion of cortisone to cortisol continues linearly for three hours, the conversion of cortisol to cortisone apparently stops at about 10 minutes. Addition of nonradioactive products to both assays (cortisol to 11oxo-reductase or cortisone to 11ß-dehydrogenase) slightly decreased the 11oxo-reductase activity as expected from product inhibition but markedly increased the apparent 11ß-dehydrogenase activity. The increase in activity is likely due to dilution of the formed [$^3$H]-cortisone preventing it from rapidly being converted back to [$^3$H]-cortisol. Indeed, adding products to both assays increased 11ß-dehydrogenase Vmax to 1433 pmol/mg protein/h, without significantly changing 11oxo-reductase Vmax (table 2 (d)). 11ß-Dehydrogenase activity in human cells was very low but it also increased when unlabeled cortisone was added‡. When assays were performed with added products human 11ß-dehydrogenase had a Km of 220nM and a Vmax of 2-4 pmol/mg protein/h; human 11oxo-reductase had a Km

of 220nM and a Vmax of 20 pmol/mg protein/h‡. Thus, 11ß-dehydrogenase activity is about 500 fold higher in Sm cells. Further, the ratio of 11ß-dehydrogenase to 11oxo-reductase activities in Sm cells is about one rather than 0.1- 0.2 found in Hu cells.

The relative forward and back rates of 11ß-HSD catalyzed reactions should reflect the intracellular NADP/NADPH ratio which depends upon cellular intermediary metabolism of glucose. Removal of glucose from the culture medium increased the extracellular ratio of [$^3$H]-cortisone to [$^3$H]-cortisol more than 10 fold (1 vs. 0.086) following incubation of Sm cells overnight with 40 nM [$^3$H]-cortisol. Starting with [$^3$H]-cortisone instead of [$^3$H]-cortisol gave similar results (1.48 vs. 0.056), indicating that equilibrium was reached, and that the decreased amount of [$^3$H]-cortisol relative to [$^3$H]-cortisone was due to the expected increase in NADP/NADPH ratio. Furthermore, when cells were incubated in glucose free medium overnight and 11ß-dehydrogenase and 11oxo-reductase activities measured in the presence of added products both rates were increased (Fig. 3). 11Oxo-reductase Km and Vmax values for cells incubated in glucose free medium overnight also are shown in Table 2 (e).

Reducing the overall rate of cellular metabolism also may be expected to change the intracellular NADP/NADPH ratio. When the temperature of cell incubation was lowered from 37° to 4° (Fig. 4), 11ß-dehydrogenase activity exceeded 11oxo-reductase activity by 8 fold at 3h, 25 fold at 6h and 100 fold at 21h. Furthermore, cortisol rather than cortisone increased the apparent 11ß-dehydrogenase activity. Values for Km and Vmax of 11ß-dehydrogenase and 11oxo-reductase determined at 4° are also shown in Table 2 (c).

Regulation of Sm 11ß-HSD: We have recently shown that treatment of human skin fibroblasts with glucocorticoids or serum withdrawal increases, while addition of insulin, 8-bromo cAMP (cAMP) or phorbol myristate acetate (PMA) decreases both 11ß dehydrogenase and 11oxo-reductase activities in parallel‡. Similar studies in Sm cell lines showed minor effects of such treatments (Fig. 5). Other hormones were also tested at several concentrations for various times and shown to be ineffective in modulating 11ß-HSD activity. These included estradiol, progesterone (alone and combined) testostersone, dihydrotestosterone and triiodothyronine. Also, cAMP (1mM) and PMA (10nM) were without effect on 11ß-HSD when added to Sm cells cultured in serum free medium.

## DISCUSSION

Early studies suggested that low affinity and/or reduced levels of receptors are responsible for the extraordinarily high circulating levels of cortisol and other steroid hormones found in some new world primates. However, comparisons of receptor binding characteristics in fibroblasts or tissues from humans, old world and new world primates revealed only about 2 fold lower affinity of GR (3,4,6), progesterone (10), and estrogen receptors (11). Inexplicably, larger differences in GR affinity were found in whole cell assays of leukocytes from squirrel and owl monkeys (1) but not in virally transformed lymphocytes from marmosets (12). The present results suggest that estimates of receptor affinity constants in new world primates may be in error due to variable binding of ligands to highly expressed 11ß-HSD resulting in overestimation of free levels in disequilibrium assays (13). On the other hand, low concentrations of all steroid receptors including those for androgens (6) were found in both tissues and cells suggesting that decreased sensitivity to the high plasma steroid levels may have occurred by down regulation of cellular receptor levels. Nonetheless, the unusual urinary excretion pattern of extremely high cortisol and very small amounts of ring A reduced metabolites found in the squirrel monkey (14) and other new world species coupled with a low metabolic clearance rate of cortisol (15) suggested that altered metabolism also may play a role in the apparent resistance to multiple steroid hormones (6).

11ß-HSD interconverts the active glucocorticoids, cortisol and corticosterone, with their biologically inactive counterparts, cortisone and 11-deoxycorticosterone, and is widely distributed amongst species and tissues (16). The function of 11ß-HSD is poorly understood although the placental enzyme is believed to regulate the availability of cortisol to the developing fetus (17,18). More recent studies have suggested that 11ß-HSD may

protect mineralocorticoid target cells (19) and Leydig cells in the testes (20) from deleterious effects of glucocorticoids by inactivating cortisol or corticosterone. However, these workers assumed that the enzyme acts unidirectionally and measured only 11ß-dehydrogenase activity in tissue homogenates (19) or immunostained 11ß-HSD protein (20). Such proposals may require reassessment in view of the present results.

We have shown that 11ß-dehydrogenase activity is 360-720 fold higher in Sm as compared to Hu genital skin fibroblasts. This is most likely the result of overexpression of 11ß-HSD protein in SM cells, since the difference in activity can be accounted for by the very large difference in Vmax values (1433 vs. 2-4 pmol/mg protein/h). The difference in Km (300-400 vs 220nM) was much smaller. Quantitation of 11ß-HSD protein in whole cells by [$^{3}$H]-cortisone binding at 4$^{o}$ has confirmed this conclusion[§]. However, the ratio of 11ß-dehydrogenase to 11oxo-reductase activities is about 0.5-1 in Sm cells (Table 2-d), whereas it is 0.1-.2 in Hu cells[†±†††]. Therefore, Sm cells not only have the capacity to inactivate cortisol more rapidly, they also are more efficient due to a shift toward oxidation of cortisol to cortisone. The latter is most likely due to a higher intracellular NADP/NADPH ratio and/or ability to regenerate the NADP-enzyme active site. The observations that removal of glucose from, or lowering the temperature of the incubation medium further increased the ratio of 11ß-dehydrogenase to 11oxo-reductase activities are in agreement with this hypothesis. Both changes should decrease the rate of NADPH generation via the pentose phosphate pathway of glucose metabolism. The shift toward cortisone formation found in Sm cells is reflected in vivo, since the plasma cortisol to cortisone ratio is considerably lower in squirrel monkeys (2:1) as compared to humans (10:1)[¶]. These results suggest that glucose metabolism may differ considerably in new world primates due perhaps to low activity of a very different insulin molecule such as found in the owl monkey (21). That the

overexpression of 11ß-HSD may be the cause of glucocorticoid resistance in squirrel monkeys is in agreement with the proposed role of this enzyme in inactivating cortisol in mineralocorticoid target tissues (19),the placenta (17,18), and the testis (20). Moreover, overexpressed 11ß-HSD could readily explain the absence of inadverent mineralocorticoid activity despite extremely high cortisol levels and the apparent decrease in cortisol affinity for otherwise normal aldosterone receptors in squirrel monkeys (5). The observation that cortisol levels are resistant to suppression by dexamethasone in squirrel monkeys(4) is in apparent conflict with a primary role of 11ß-HSD in cortisol resistance in these animals,since dexamethasone appears to be a poor substrate for 11ß-HSD. However, this has not been unequivocaly shown. Further, unsuppresed cortisol levels may be due to some degree of autonomy of squirrel monkey adrenals rather than glucocorticoid resistance at the hypothalamic/pituitary level. Infact, despite similar levels of ACTH,cortisol production rates are 6 fold higher in squirrel monkeys than in cynomolgus monkeys (15).

In addition to inactivating cortisol by converting it to cortisone, binding of cortisol to 11ß-HSD will further reduce the intracellular pool of free cortisol that is available to GR. We estimate from whole cell binding studies using [$^3$H]-cortisone and [$^3$H]-dexamethasone as ligands that the level of 11ß-HSD is at least 100 fold higher than the concentration of GR in Sm fibroblasts[§]. Interestingly, we also have found that other steroids including progesterone and testosterone compete for binding, inhibit cortisol-cortisone interconversion and act as substrates for 11ß-HSD in Sm cells. These results suggest that overexpression of 11ß-HSD in squirrel monkeys may contribute to the high plasma levels of multiple steroid hormones. In keeping with these findings is the observation that unlike humans the

plasma levels of androstenedione, the product of 11ß-HSD action on testosterone, are higher than those of testosterone in the squirrel monkey (6).

In contrast to human skin fibroblasts, 11ß-HSD in Sm cells does not seem to be hormonally regulated since treatment with various steroid hormones, thyroid hormone, cAMP and insulin were without effect. It is possible that the increase in 11ß-HSD activity in human skin fibroblasts caused by dexamethasone treatment results from derepression rather than enhanced transcription of the 11ß-HSD gene. In this event 11ß-HSD in Sm cells may not be repressed which would explain the lack of response to glucocorticoid treatment. Interestingly, this appears to be the case with the aromatase gene in strains of chickens with the henny-feathering trait (19). Glucocorticoids up regulate expression of the aromatase gene in skin fibroblasts from normal chickens but in affected strains the gene is highly expressed and aromatase activity can not be increased by glucocorticoid treatment. The failure of human insulin to down regulate 11ß-HSD in Sm cells must be interpreted with caution since the Sm insulin receptor has not been characterized. Elucidation of the molecular basis for the lack of regulation of 11ß-HSD will be of great interest and awaits cloning and analysis of the Sm gene.

We can only speculate on the evolutionary pressures that resulted in the dramatically elevated plasma cortisol levels in some new world primates and not in others. Increased expression of 11ß-HSD leading to high cortisol levels may have accompanied the genetic drift towards an ineffective insulin molecule. In this event, increased cortisol production may be viewed as a compensatory response since optimal glucocorticoid effects require the presence of insulin in several systems. Alternatively, development of an endocrine system with high circulating but relatively normal intracellular levels of cortisol may have provided protection

against parasitic organisms that are susceptible to the killing effects of glucocorticoids. Parasitic organisms containing receptor-like binding proteins for, and are responsive to, estradiol (23) and progesterone (24) have been reported. A general increase in 11ß-HSD activity in Sm cells could decrease negative feedback in the hypothalamic-pituitary-adrenal axis (HPA), resulting in increased cortisol production (15) and high circulating cortisol levels that may be lethal to parasites while host cells are spared. The markedly lowered affinity and binding capacity of CBG (25,26), and reduced clearance of cortisol by hepatic 5a- and 5ß-reduction of ring A (14) also contribute to increased activity of plasma cortisol. This scenario could explain the selective appearance of high cortisol levels in some new world primates and rodents such as the guinea pig (27) but not in others. It will be of interest to determine whether 11ß-HSD is also overexpressed in humans who are resistant to high plasma cortisol levels (1,2).

Clearly, the squirrel monkey can be regarded as an extreme situation in expression and function of 11ß-HSD. As previously suggested, this enzyme likely plays a key role in modulating glucocorticoid action at the target cell level in human and other species. The demonstration that changing glucose levels markedly influences the cellular cortisol:cortisone ratio suggests that 11ß-HSD may provide a link between regulatory elements of the HPA axis and food intake.

## REFERENCES

1. Chrousos, G.P., Loriaux, D.L., Brandon, D., Tomita, M., Vingerhoeds, A.C.M., Merriam, G.R., Johnson, E.O., and Lipsett., M.B. (1983) J Steroid Biochem **19**(1), 567-575.

2. Iida, S., Gomi, M., Moriwaki, K., Itoh, Y., Hirobe, K., Matsuzawa, Y., Katagiri, S., Yonezawa, T., and Tarui, S. (1985) J Clin Endocrinol Metab. **60**:967-971.

3. Chrousos, G.P., Lorianx, D.L., Tomita, M., Brandon, D., Renquist, D., Albertson, B., and Lipsett, M.B. (1986) In: Advances in experimental medicine and biology. Chrousos, G.P., Lorianx, D.L., and Lipsett, M.B. (eds) Plenum Press, N.Y., **196**, 129-144.

4. Chrousos, G.P., Renquist, D., Brandon, D., Eil, C., Pugeat, M., Vigersky, R., Cutler Jr., G.B., Loriaux, D.L., and Lipsett, M.B., (1982) Proc Natl Acad Sci USA **79**, 2036-2040.

5. Chrousos, G.P., Loriaux, D.L., Brandon, D., Shull, J., Renquist, D., Hogan, W., Tomita, M., and Lipsett, M.B. (1984) Endocrinology **115**, 25-32.

6. Siiteri, P.K. (1986) In: Advances in experimental medicine and biology. Chrousos, G.P., Lorians, D.L, and Lipsett, M.B. (eds) Plenum Press N.Y., **196**, 279-289.

7. Bradford, M.M. (1976) Analyticol Biochem. **72**, 248-254.

8. Phillips, D.M., Lakshmi, V., and Monder, C. (1989) Endocrinology **125**, 209-216.

9. Schulz, W., Lichtenstein, I., Siebe, H., and Hierholzer, K. (1989) J Steroid Biochem. **32**(4), 581-590.

10. Chrousos, G.P., Renquist, D., Brandon, D., Barnard, D., Fowler, D., Loriaux D.L, and Lipsett, M.B. (1982) J Clin Endocrinol Metab. **55**(2), 364-368.

11. Chrousos, G.P., Brandon, D., Renquist, D.M., Tomita, M., Johnson, E., Loriaux D.L., and Lipsett M.B. (1984) J Clin Endocrinol Metab. **58**(3), 516-520.

12. Tomita, M., Brandon, D.D., Chrousos, G.P., Vingerhoeds, A.C.M., Foster, C.M., Fowler, D., Loriaux, D.L., and Lipsett, M.B. (1986) J Clin Endocrinol Metab **62**(6), 1145-1154.

13. Siiteri, P.K. (1984) Science **223**, 191-193.

14. Setchell, K.D.R., Chua, D.S., and Himsworth, R.L. (1977) J Endocr **73**, 365-375.

15. Cassorla, F.G., Albertson, B.D., Chrousos, G.P., Booth, J.D., Renquist, D., Lipsett, M.B., and Loriaux, D.L. (1982) Endocrinology **111**(2), 448-451.

16. Monder, C., and Shackleton, C.H.L. (1984) Steroids **44**, 383.

17. Murphy, B.E.P., and Branchaud, C.T.L. (1983) Curr Top Exp Endocrinol **5**, 197.

18. Baggia, S., Albrecht, E.D., and Pepe, G.J. (1990) Endocrinology **126**(5), 2742-2748.

19. Funder, J.W., Pearce, P.T., Smith, R., and Smith, A.I. (1988) Science **242**, 583-585.

20. Monder, C. (1990) In: Steroid formation, degradation, and action in peripheral tissues. Castagnetta, L., d'Aquino, S., Labrie, F., and Bradlow, H.L. (eds) The New York Academy of Sciences, N.Y., **595**, 26-39.

21. Seino, S., Steiner, D.F., and Bell, G.I. (1987) Proc Natl Acad Sci USA **84**, 7423-7427.

22. Leshin, M., Noble, J.F., George, F.W., and Wilson, J.D. (1983) J Steroid Biochem. **18**, 33-39.

23. Clemons, K.V., Feldman, D., Stevens, D.A. (1989) J General Microbiology **135**, 1607-1617.

24. Clemons, K.V., Stover, E.P., Schor, G., Stathis, P.A., Chon, K., Tokes, L., Stevens, D.A., and Feldman, D. (1989) J Biological Chemistry **264**, 11186-11192.

25. Pugeat, M.M., Chrousos, G.P., Nisula, B.C., Loriaux, D.L., Brandon, D., and Lipsett, M.B. (1984) Endocrinology **115**, 357-361.

26. Klosterman, L.L., Murai, J.T., Wiener, S.T., Levine, S.G., and Siiteri, P.K. (1986) Endocrinology **118**:424-434.

27. Kraft, N., Hodgson, A.J., and Funder, J.W. (1979) Endocrinology **104**:344-349.

FOOT NOTES

* This work was supported by NIH grant CA-27702

† To whom correspondence should be directed

Phone: (415) 476-2719

Fax: (415) 753-3271

Address: Reproductive Endocrinology Center
University of California San Francisco
San Francisco, California 94143

‡ Muhammad M. Hammami and Pentti K. Siiteri. Regulation 11ß-hydroxysteroid dehydrogenase (11ß-HSD) in human skin fibroblasts: Enzymatic modulation of glucocorticoid action. Submitted.

§ Muhammad M. Hammami, Jean-Louis Vigne and Pentti K. Siiteri. Specificity, steroid binding and affinity labeling of 11ß-Hydroxy steroid dehydrogenase (11ß-HSD). Role in general steroid resistance. Submitted.

¶ Pentti K. Siiteri. Unpublished observations.

The abbreviations used are: 11ß-Hydroxysteroid dehydrogenase, 11ß-HSD; Sm, Squirrel Monkey; CBG, cortisol binding blobulin; GR, glucocorticoid receptors; AR, aldostorsone receptors; DME, Dulbecco's modified Eagle's medium; CS, calf serum; TLC, thin layer chromatography; Rh, rhesus monkey; Hu, human.

## TABLE 1

11ß-HSD activity in Sm, Rm and Hu cell lines

| Cell line | 11ß-reductase | 11ß-oxidase |
|---|---|---|
| Sm P2F | 140 | 3.4 |
| Sm 1-F | 87 | 33 |
| Sm B7-F | 160 | 6 |
| Sm B1-La | 124 | 6.4 |
| Sm 5-La | 73 | 37 |
| Sm KSK | 86 | 56 |
| Sm Fat-02 | 37 | 2.27 |
| Sm Fat-04 | 30 | 1 |
| | | |
| Rhm K-$F_3$ | 10 | 0.7 |
| Rhm 2-F | 10 | 1.1 |
| Rhm La-1 | 3.4 | 0.2 |
| 560D | 1.7 | UD |
| HS 555SK | 1.95 | UD |
| HS681 | 6.3 | UD |
| 26D | 5 | 0.85 |
| HS547 | 5 | 3.3 |
| 628D | 17 | 0.5 |

Results are typical of two experiments. Activities were determined at 30 minutes using 40nM substrates. Values are the mean of duplicate assays with errors typically less than 5% and are in pmol/mg protein/h. UD, undetected.

# TABLE 2

Kinetic studies of 11ß-HSD in Sm skin fibroblasts incubated under different conditions.

| | 11ß-dehydrogenase | | 11oxo-reductase | |
|---|---|---|---|---|
| | Km | Vmax | Km | Vmax |
| a) * | 428±67 | 460±46 | 315±33 | 1329±72 |
| b) * | 639±145 | 709±9 | 506±40 | 1978±91 |
| c) † | 1721±244 | 113±12 | 3328±703 | 109±18 |
| | 2859±465 | 133±20 | 3007±224 | 62±3.4 |
| d) + | 403±25 | 1433±70 | 734±60 | 1940±200 |
| e) § | - | - | 493±30 | 66±40 |

Km and Vmax values were determined using duplicate results obtained at eight substrate concentrations (42 to 5600nM). Incubation was in DME at 37° for 10 minutes unless indicated otherwise. Km and Vmax are in nM and pmol/mg protein/h ± S.E., respectively.

* Passage 5 and 25, respectively. †Incubation was at 4° for 3 and 6 hrs, respectively. +Unlabeled product was added to the assay medium. §Cells were preincubated in glucose free medium overnight.

Fig. 1

11ß-HSD saturation plots (typical of four experiments). Both (a) and (b) were carried out for ten minutes. a) 11oxo-reductase, b) 11ß-dehydrogenase.

Fig. 2

11ß-HSD time course (typical of three experiments). Each symbol represents the average of duplicate assays with errors smaller than symbol size a) 11oxo-reductase, cells were incubated with 190nM [$^3$H]-cortisone with or without 190 µM cortisol. b) 11ß-dehydrogenase cells were incubated with 190nM [$^3$H]-cortisol with or without 190 µM cortisone.

Fig. 3

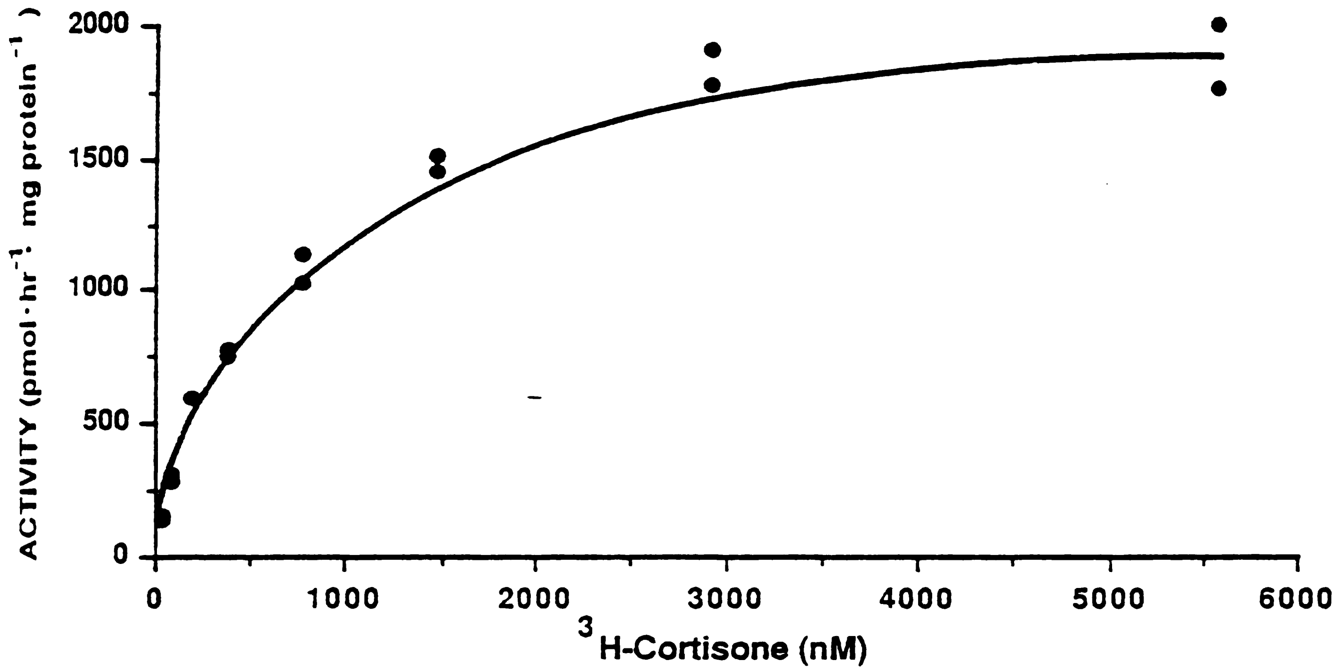
11ß-HSD activity in glucose free medium. Bars represent the average of duplicate assays. Cells were incubated in glucose free-DME at 37° overnight. Substrates were added (with or without additions) in glucose free-DME. The assays were carried out for the indicated times. a) 11oxo-reductase; cells were incubated with 40nM [$^3$H]-cortisone with or without 4 µM cortisol. b) 11ß-dehydrogenase; cells were incubated with 40nM $^3$H-cortisol with or without 4µM cortisone.

Fig. 4

11ß-HSD time course at 4° (typical of two experiments). Bars represent the average of duplicate assays. Cells were incubated at 4° for 2 hrs, substrates were added (with or without additions) in cold (4°) DME and assays were carried out for the indicated times. a) 11oxo-reductase; cells were incubated with 240nM [$^3$H]-cortisone with or without 48µM cortiosl. b) 11ß-dehydrogenase; cells were incubated with 230nM [$^3$H]-cortisol with or without 46µM cortisone.

Fig. 5

11ß-HSD regulation (typical of two experiments). Bars represent the average of duplicate assays. Cells were incubated for 113 hrs. at 37° with one of the following media: 10% stripped calf serum (10% SCS) with or without 10μM Dexamethasone (Dex), 4mg/mL human albumin (SF) with or without 10μM insulin (Ins). The medium was changed every 48 hrs. After washing three times with DME, substrates were added in DME and the assays were carried out for 10 minutes. a) 11oxo-reductase; [$^3$H]-cortisone concentration was 55nM. b) 11ß-dehydrogenase; [$^3$H]-cortisol concentration was 40nM.



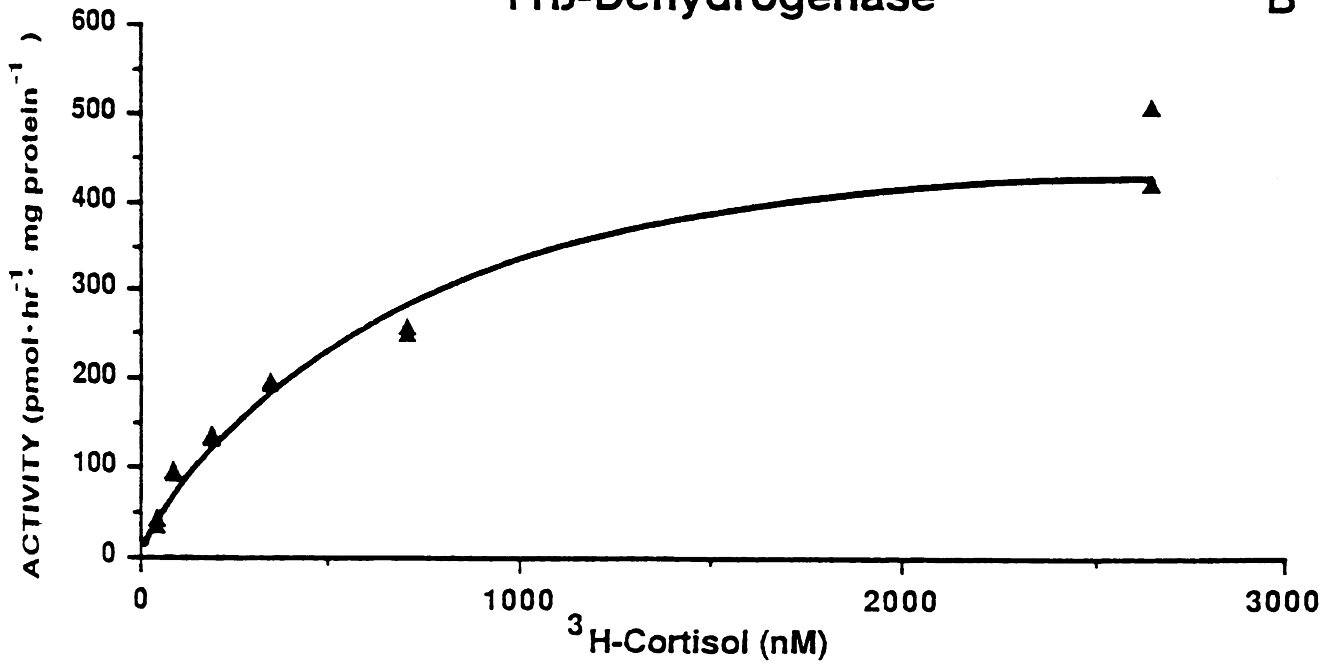
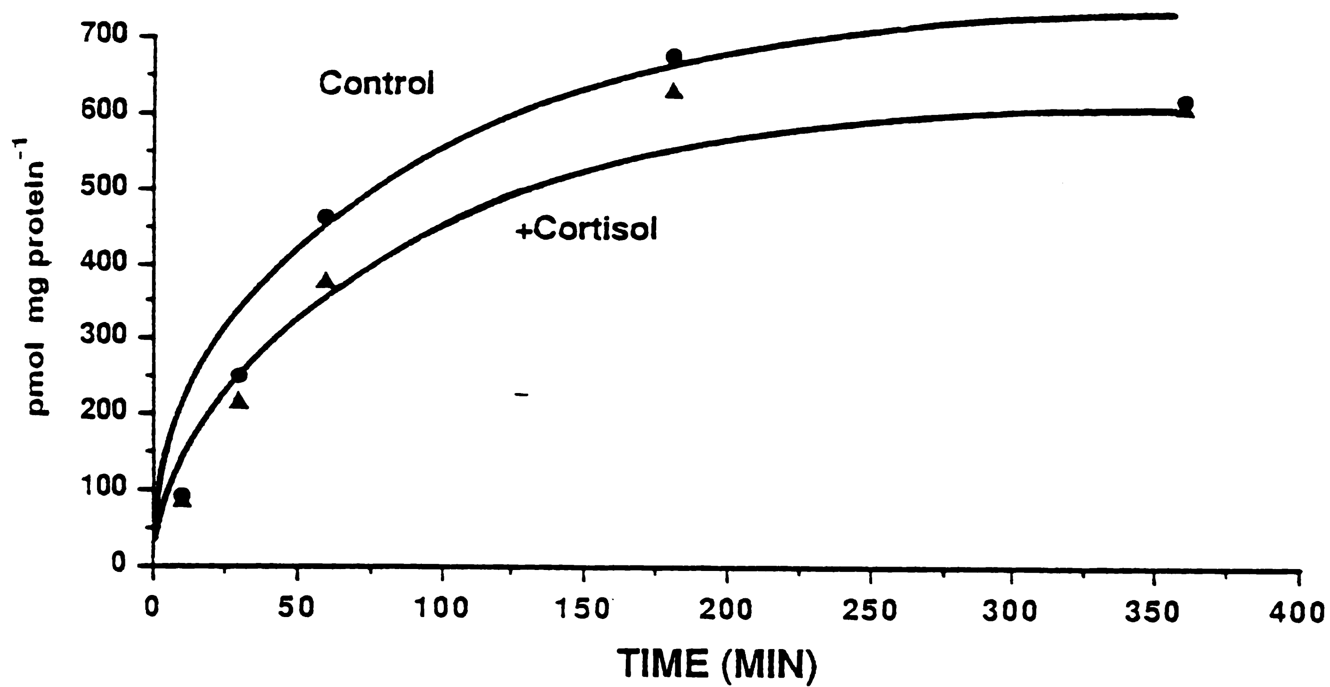




Fig 1



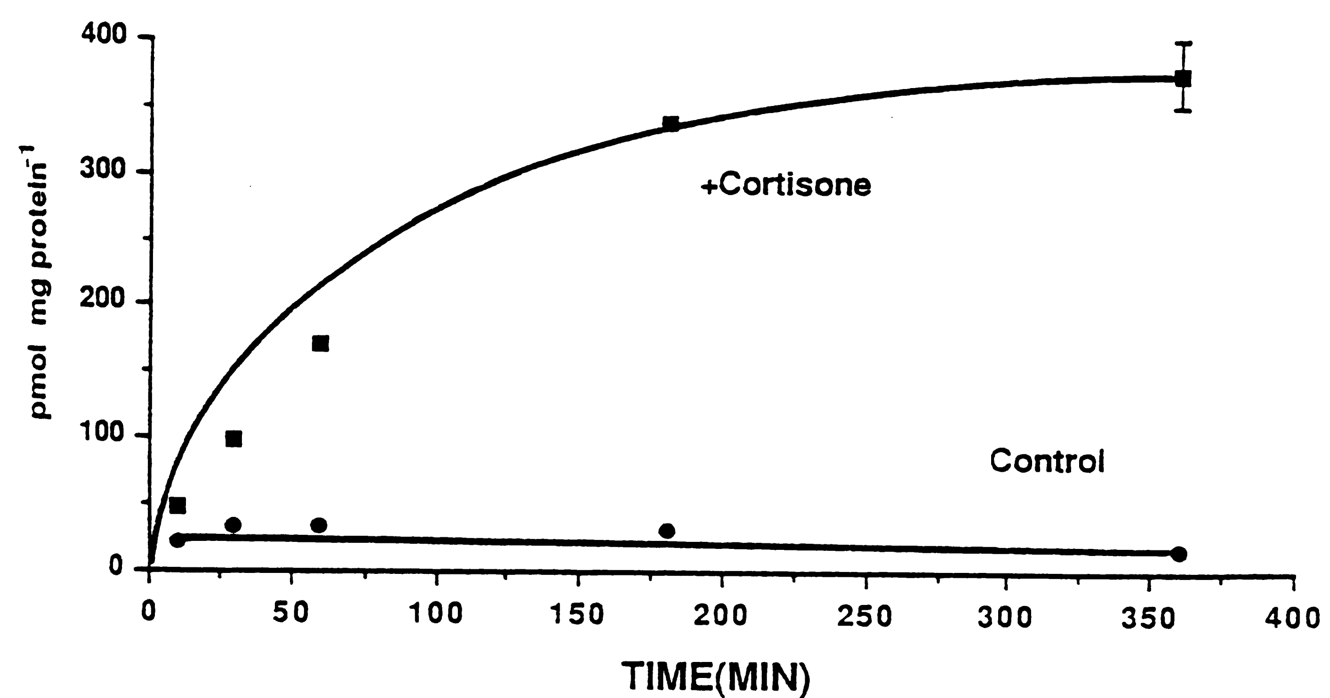
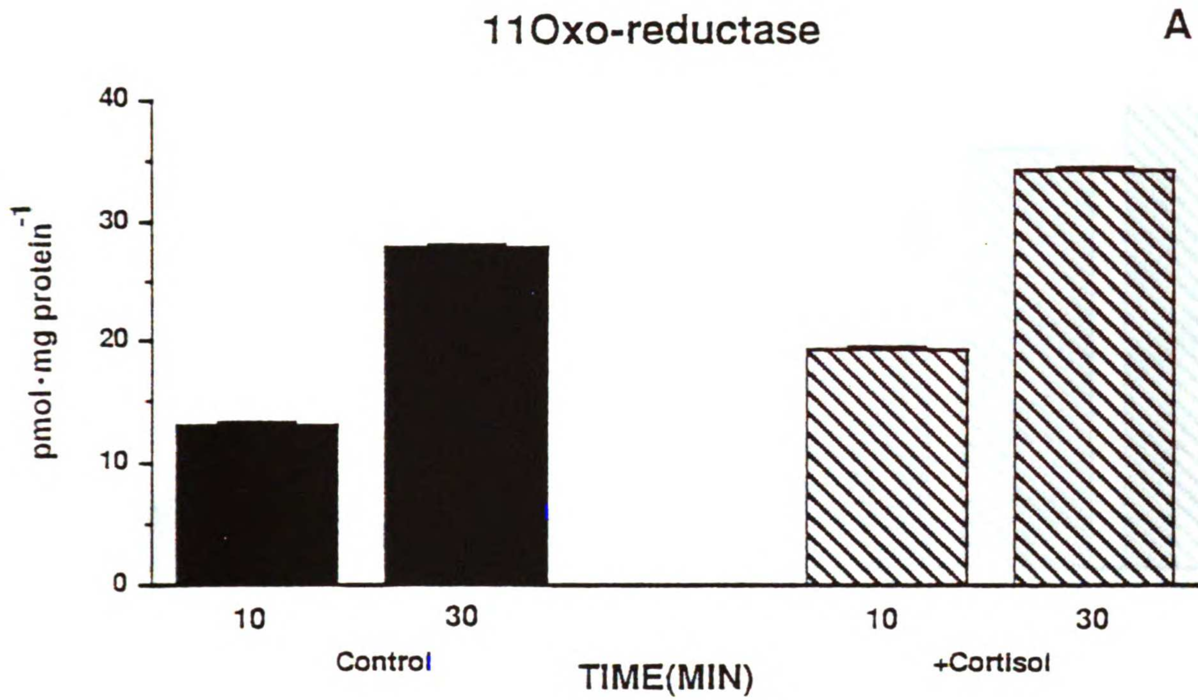




Fig 2



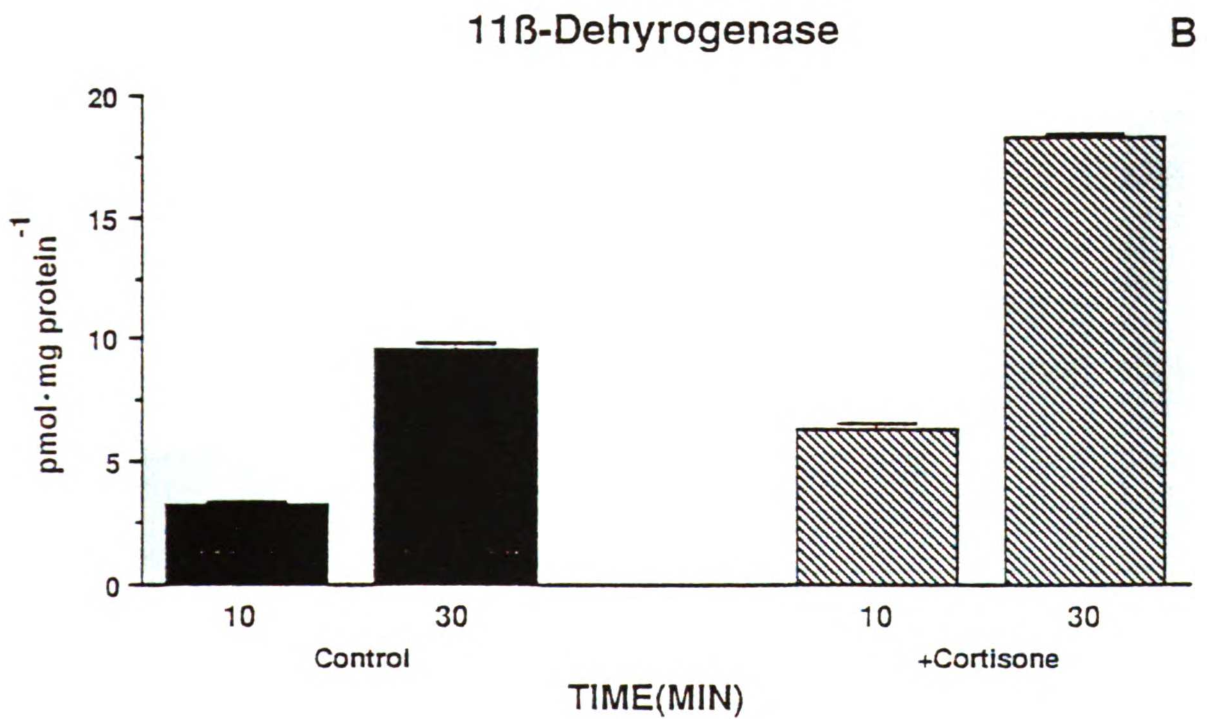
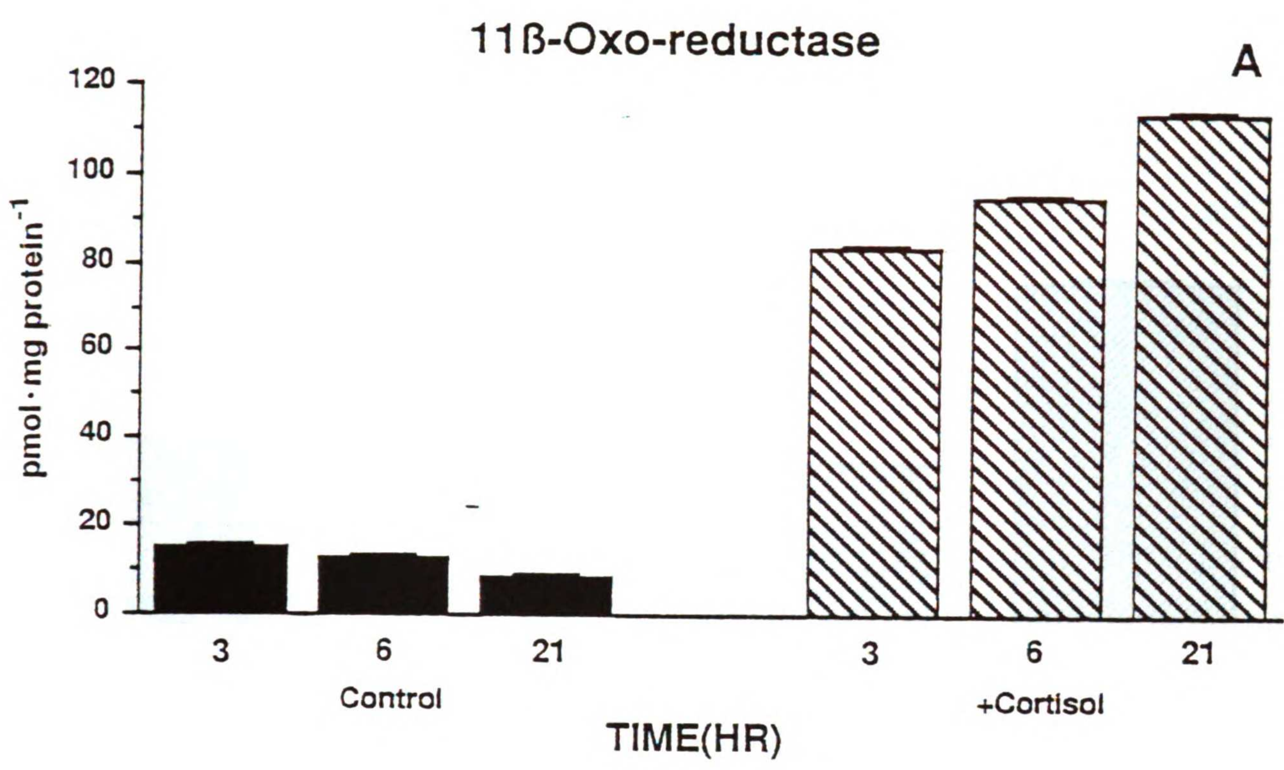




Fig 3



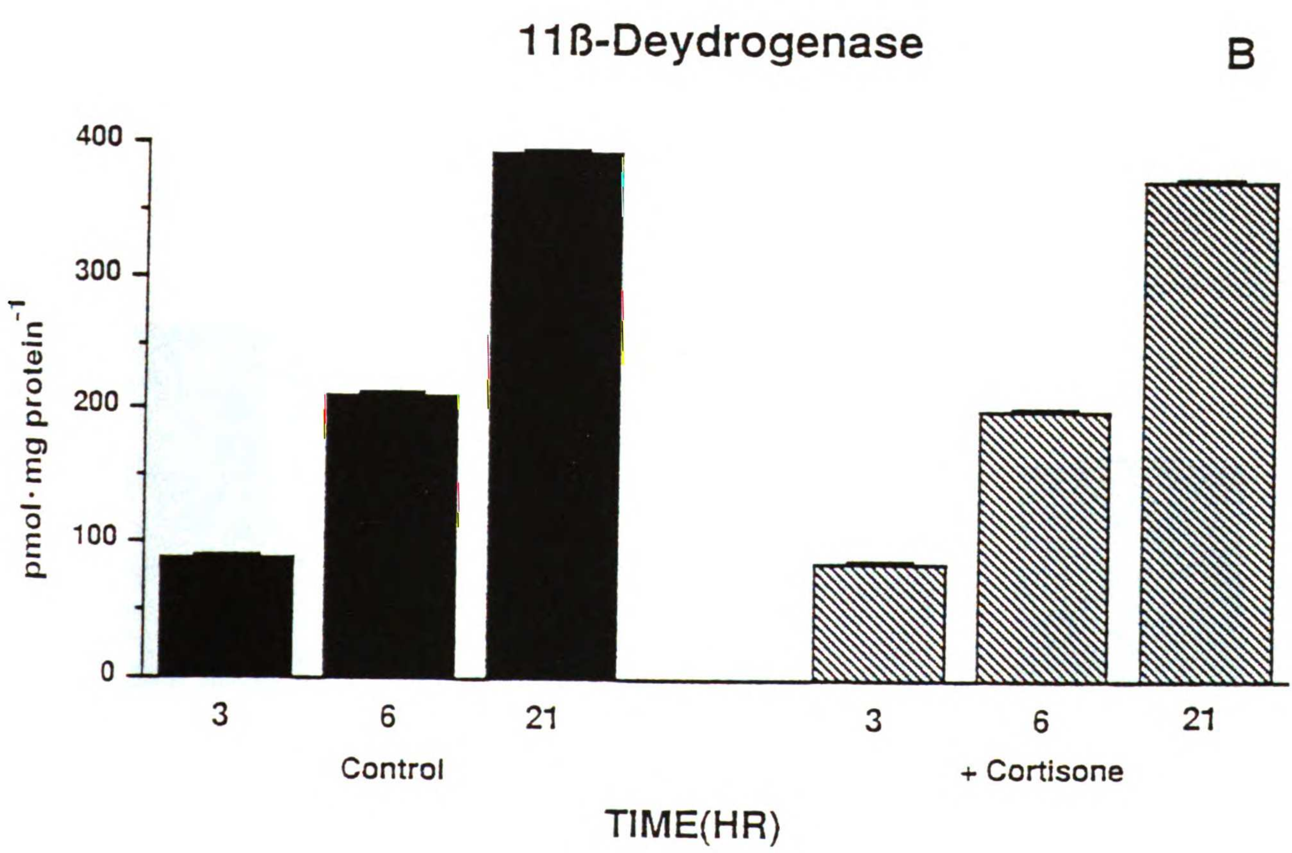
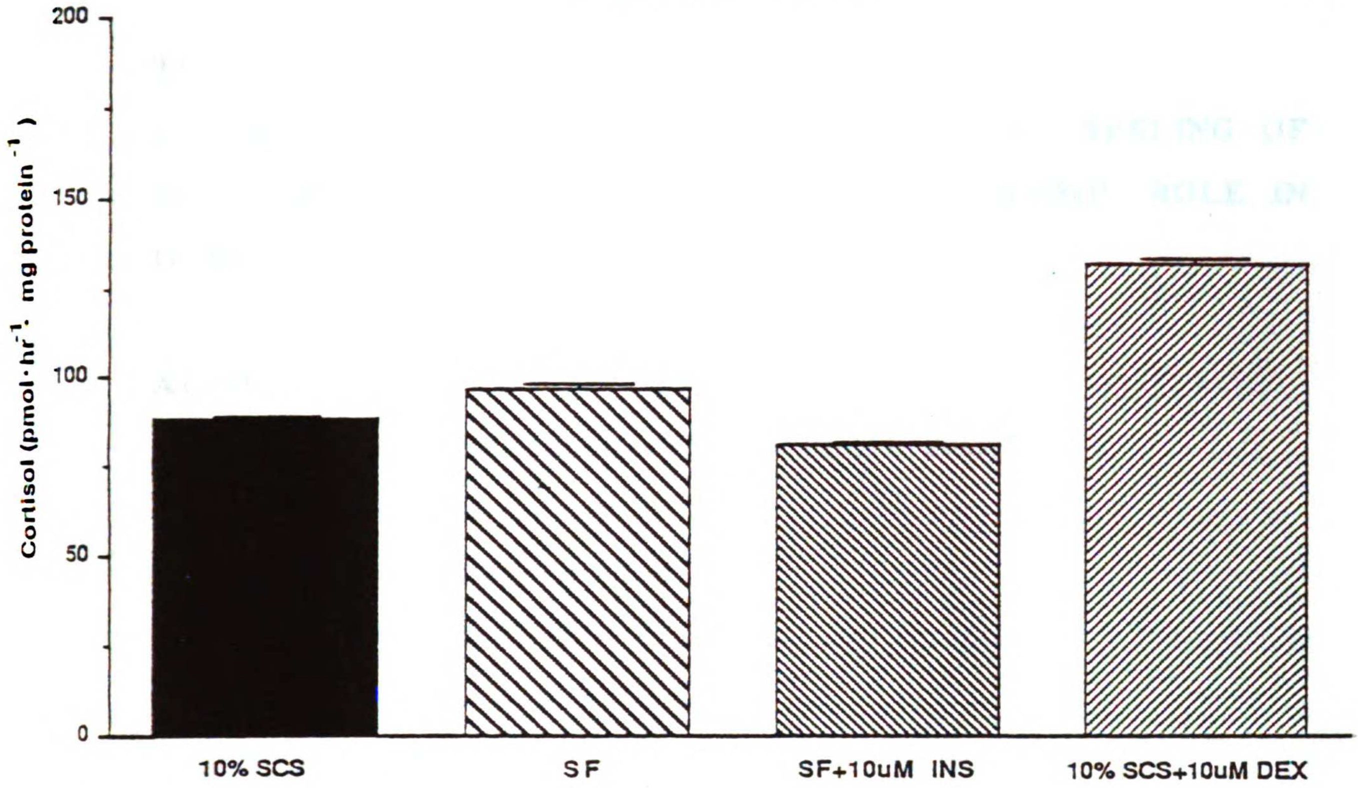




Fig 4



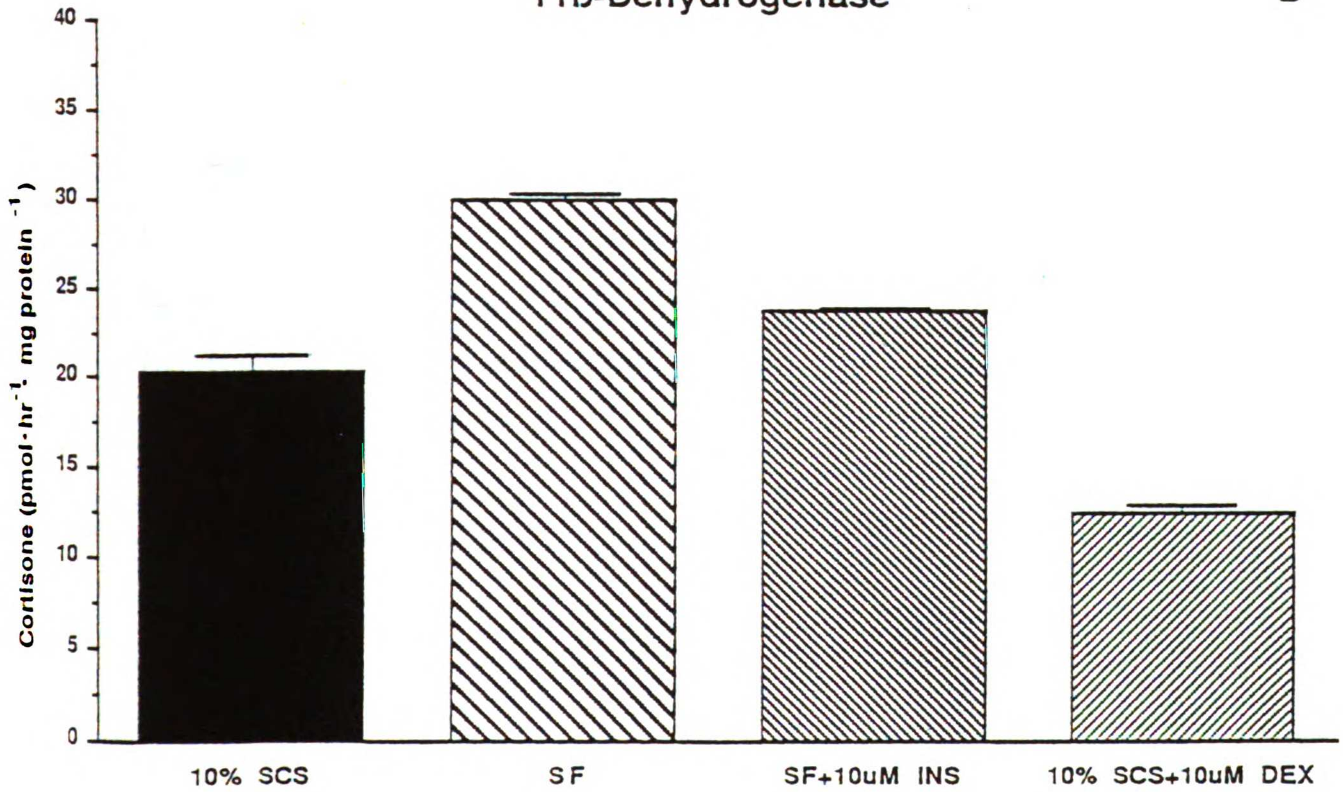




Fig 5

**TITLE:**

**SPECIFICITY, STEROID BINDING AND AFFINITY LABELING OF 11ß-HYDROXY STEROID DEHYDROGENASE (11ß-HSD): ROLE IN GENERAL STEROID RESISTANCE***

**AUTHORS:**

Muhammed M. Hammami, Jean-Louis Vigne and Pentti K. Siiteri+

Reproductive Endocrinology Center

University of California, San Francisco

San Francisco, California 94143

**Running title:** 11ß-Hydroxysteroid dehydrogenase and general steroid resistance.

* **Supported by** NIH grant CA-27702

+ **Address correspondence to:**

Pentti K. Siiteri, Ph.D.

Reproductive Endocrinology Center

University of California San Francisco

San Francisco, California 94143

Phone: (415) 476-2179

Fax: (415) 753-3271

**Key words**: 11ß-hydroxysteroid dehydrogenase; specificity; affinity labeling; steroid resistance; cortisone binding.

## ABSTRACT

We examined the ability of several steroid hormones to inhibit 11ß-HSD activity in squirrel monkey (Sm) cells. Inhibition of 11-oxo-reduction of [$^3$H]cortisone was observed with the following potency: 11oxy-progesterone > cortisone > progesterone > 11ß-hydroxyprogesterone > testosterone > cortisol > Ru486 = dexamethasone. Inhibition of 11ß-oxidation(11ß-dehydrogenase) of [$^3$H]cortisol also occurred with 11ß-hydroxyprogesterone > cortisol > progesterone > testosterone> dexamethasone whereas cortisone and 11-oxy-progesterone were stimulatory rather than inhibitory. Scatchard analysis of [$^3$H]cortisone binding to Sm cells at 4° revealed a Kd around 400nM and total binding sites of 55 pmol/mg protein. The potency of steroid hormones in inhibiting [$^3$H]cortisone binding closely followed the pattern of inhibition of 11-oxo-reductase activity. Further, affinity labeling of cells with [$^3$H]dexamethasone mesylate showed a 34 KDa band that was reduced when excess cortisone, progesterone or testosterone but not triamcinolone was added. Furthermore, cortisone, androstenedione and progesterone had similar potencies in inhibiting [$^3$H]progesterone or [$^3$H]- androstenedione binding to and metabolism by Sm cells. We conclude that 1) Sm fibroblasts overexpress an apparently normal 11ß-HSD protein; 2) 11ß-HSD catalyzes the interconversion of several active and inactive steroid hormones and therefore it may be involved in multiple steroid resistance found in squirrel monkeys; 3) glucocorticoid activity may be modulated by other steroid hormones via competition for 11ß-HSD; and that 4) affinity labeling with dexamethasone or cortisone mesylate provides an efficient way to study this enzyme at a molecular level.

## INTRODUCTION

Resistance to steroid homones continues to attract attention due to the insight that may be gained on the mechanism of their action. Recently, we have shown that 11ß-HSD activity is about hundred fold higher in squirrel monkey (Sm) as compared to human (Hu) skin fibroblasts and suggested that Sm cells overexpress an apparently normal 11ß-HSD protein (1). Furthermore, in contrast to Hu or rhesus monkey (Rh) cells, the relative oxidase and reductase activities of 11ß-HSD in Sm cells favors the formation of cortisone rather than cortisol. These results suggested that 11ß-HSD may have a primary role in the glucocorticoid resistance found in this species and other new world primates (1).

The high activity of 11ß-HSD in Sm fibroblasts provided a convenient model to further characterize this enzyme in intact, homogeneous cells that can be maintained in continuous culture. The specificity of 11ß-HSD is of particular interest since several new world primates are resistant not only to cortisol but to testosterone and progesterone as well (2). These primary hormones also are inactivated when oxidized to androstenedione or reduced to 20$\alpha$-hydroxy-pregn-4ene-3one, respectively by reactions that theoretically at least, can be catalyzed by 11ß-HSD.

In this report we demonstrate that 1) testosterone, androstenedione and progesterone, among other steroids competitively inhibit the interconversion of cortisol and cortisone, 2) these steroids are converted to their corresponding reduced or oxidized counterparts by Sm cells; and 3) their conversion is inhibited by each other and by cortisone. We also found that at 4° cortisone displays specific binding with a Kd of 400nM to a total binding sites of 55 pmol/mg protein in Sm but not in human cells. That these binding sites are 11ß-HSD was shown by the

following 1) various steroids inhibited cortisone binding in an order of potency similar to their inhibitory effects on 11ß-HSD activity 2) affinity labeling of SM (but not Hu) cell proteins with dexamethasone mesylate produced a 34 KDa band which could be blocked by addition of excess cortisone and to a lesser extent 11oxy-progesterone, progesterone and testosterone but not triamcinolone or estradiol. Together these results confirm that 11ß-HSD is highly overexpressed in Sm cells and demonstrate that the enzyme has broad substrate specificity. The data strongly suggest that 11ß-HSD may be responsible for the elevated plasma levels of multiple steroids in new world primates.

## RESULTS

Inhibition of 11ß-reduction(11-oxo-reductase) of [$^3$H]cortisone and 11ß-oxidation(11ß-dehydrogenase) of [$^3$H]cortisol by other steroids: Sm cells (Sm 51a, Labial skin fibrolasts) were incubated either with 40 nM [$^3$H] cortisone or [$^3$H] cortisol with or without 0.1 to 1000 fold excess unlabeled steroids for 10 minutes and remaining substrate and product were then determined. The results are summarized in Table I. It appears that 11-oxy-progesterone is a better inhibitor of 11-oxo-reductase activity than cortisone. Similarly 11ß-hydroxyprogesterone is a better inhibitor of 11ß-dehydrogenase activity than cortisol. itself. Progesterone and testosterone are efficient inhibitors of 11ß-HSD activities whereas the synthetic glucocorticoids are not. Inhibition of 11-oxo-reductase activity by cortisol and of 11ß-dehydrogenase activity by higher concentrations of cortisone may be allosteric or competitive. The paradoxical increase of 11ß-dehydrogenase activity found with lower concentrations of cortisone or 11-oxy-progesterone is likely the result of greater inhibition of 11-oxo-reductase than of 11ß-dehydrogenase activities (1, 3). The effect of high concentrations of other steroids on 11ß-HSD activities was also

examined. The following results were obtained when competitors were added at 1,000 fold excess and are expressed as percent remaining activity of 11ß-oxo-reductase and 11ß-dehydrogenase, respectively: dihydrotestosterone; (23, 91); triamcinolone (82, 58); R5020 (19, 93); R1881 (20, 69) dexamethasone mesylate (116, 70); estradiol (83, - ); aldosterone (30, 8); estrone (40, 74); and androstenedione (62, 107).

Binding of [$^3$H]cortisone to 11ß-HSD: Since the much higher 11ß-HSD activity in Sm than in human cells can be accounted for by a markedly increased Vmax (1), we attempted to quantitate 11ß-HSD protein by examining the binding of [$^3$H]cortisone to Sm cells at 4°. Table 2 demonstrates the validity of the binding assay. Three washes of cells at 4° decreased the non specific to specific binding ratio to less than 15% without decreasing specific binding values. Fig. 1 shows a representitive [$^3$H]cortisone binding saturation plot and its Scatchard transformation. Pooling the results obtained with three separate passages of cells gave a Kd of 400 nM (range: 288-465) and a total binding capacity of 55 pmol/mg protein (range: 41-64). By way of comparison [$^3$H]DEX binding to glucocorticoid receptors in Sm cells exhibits a Kd of 6-10 nM and total binding site of 0.1-0.2 pm/mg protein. Under identical conditions, no saturable binding of [$^3$H]cortisone was detected in human cells (HS681) which have only about 1% of the 11ß-HSD activity found in Sm cells. These results indicate that [$^3$H]cortisone is bound to 11ß-HSD. The steroid specificity of [$^3$H]cortisone binding was studied in Sm cells using 140 nM [$^3$H]cortisone and competitors at 0.02 to 200 fold excess. From results shown in Table 3 it is clear that the specificity of the binding does not fit known steroid binding proteins such as the glucocorticoid receptor or serum corticosteroid binding globulin. Furthermore, the potency of effective steroids in inhibiting the binding of [$^3$H]cortisone closely follows their potency in inhibiting

11ß-reductase activity (Table 1). These data also strongly suggest that [$^3$H]cortisone is bound to 11ß–HSD in Sm cells. In addition to the steroids in Table 3 other steroids were examined at 200 fold excess and the following results were obtained: estrone, (51); estradiol, (51); R5020, (15); R1881, (17); dexamethasone mesylate (DM), (46); and dehydroepiandrosterone, (12).

Despite being a weak competitor of [$^3$H]cortisone binding and a weak inhibitor of 11ß-HSD activity, DM was successfully used to affinity label 11ß-HSD. Human or Sm cells in DME were incubated with 200nM [$^3$H]DM ± 200µM competitors at 37° for 1h. Cellular proteins were separated by SDS-PAGE as described in methods and flurography and densitometry were then performed. Fig. 2 depicts the results. A 34 KDa band was found in Sm ,but not in Hu cells, that was reduced by excess cortisone, 11 oxy-progesterone, progesterone and to a lesser extent testosterone but not triamcinolone.

Progesterone and androstenedione binding to and metabolism by Sm cells: The results demonstrating that progesterone, testosterone and androstenedione inhibit both 11ß-HSD activity and substrate binding suggested that these steroids also may be substrates of 11B-HSD. Indeed, Sm cells reduced [$^3$H]androstenedione and [$^3$H]progesterone at a rate about 10% of 11-oxo-reduction of [$^3$H]cortisone. Similarly, the rate of oxidation of [$^3$H]testosterone is about 10% of the rate of 11ß-oxidation (11ß-dehydrogenase) of [$^3$H]cortisol. The reduction of [$^3$H]progesterone had a Km of 2930 nM and a Vmax of 222 pmol/mg protein/h, whereas the reduction of [$^3$H]androstenedione had a Km of 3000 nM and a Vmax of 141 pmol/mg/h. Further, progesterone, testosterone and cortisone had similar potency to each other in inhibiting the reduction of [$^3$H]androstenedione or [$^3$H]progesterone (Fig. 3). Similarly these steroids inhibited the binding of [$^3$H]-

androstenedione or [$^3$H]progesterone to Sm cells to a similar degree to each other (Fig. 4 and data not shown). Combined, these data strongly support the view that 11ß-HSD has broad substrate specificity.

-

## DISCUSSION

Until about 10 years ago interest in steroid resistance was directed to defects in androgen dependent development of the male phenotype and to the failure of glucocorticoids to kill lymphoid tumor cells. Since then the term resistance has been applied to quite different situations where the plasma levels of various steroid hormones are greatly elevated but normal functions are maintained. Early attempts to explain the latter phenomenon focused upon possible steroid receptor abnormalities similar to those which now have been clearly shown to be associated with androgen resistance. For example, the high cortisol levels in new world primates (4) and humans (5-6) have been ascribed to low affinity glucocorticoid receptors (7) which theoretically at least, would elevate circulating cortisol levels by a secondary increase in cortisol secretion (8). High circulating levels of progesterone (2) and testosterone in new world primates have been explained on the same basis even though blood androgen levels are not elevated above the normal range in human androgen resistant states associated with receptor defects. The elevated levels of many steroid hormones including 1, 25 hydroxy vitamin D3 found in some but not all new world primates would require simultaneous mutations in many receptor proteins. Since this is highly unlikely we have sought an alternative explanation such as involvement of a steroid metabolizing enzyme that posseses broad substrate specificity. Here, we show that 11ß-HSD fulfills this role.

The inhibition of 11ß-oxidation(11ß-dehydropgenase) of cortisol by other steroids has been studied earlier (9, 10). Lopez-Bernal et al, using human placental and decidual microsomes, found that 11ß-hydroxyprogesterone, progesterone, testosterone or dexamethasone can inhibit 11ß-dehydrogenase activity when used in

10 fold excess of cortisol; inhibition varied significantly between placental and decidual preparations (9). Murphy et al, using whole tissue minces of human placenta, studied a variety of 11-hydroxylated steroids and concluded that 11ß- and 11α-hydroxyprogesterone are several fold better inhibitors of 11ß-dehydrogenase than cortisol (10). During preparation of this manuscript Baggia et al reported that progesterone is a better inhibitor of 11ß-dehydrogenase activity than cortisone and that the inhibition is competitive as deduced from Dixon plot analysis (11). Our data, using intact Sm skin fibroblasts confirms these observations and extends the spectrum of inhibitors of both 11ß-dehydrogenase and 11-oxo-reductase activities. Further, we show that the inhibition rather than being allosteric, is due to competition for the active site of the enzyme since a variety of steroids inhibited binding of [$^3$H]cortisone in Sm cells with an order of potency that is similar to their order of potency in inhibiting 11-oxo-reductase activity. Furthermore, we show that Sm cells actually metabolize progesterone, testosterone and androstenedione to their corresponding oxidized or reduced products; the ratio of testosterone oxidation to androstenedione reduction is similar to the ratio of 11ß-dehydrogenase to 11-oxo-reductase activities using cortisol and cortisone as substrates. Moreover, reduction of androstenedione to testosterone was inhibited by androstenedione, progesterone and cortisone which also inhibited its binding in Sm cells. The same results were obtained when progesterone was used instead of androstenedione. These data strongly argue that 11ß-HSD is active on a rather wide spectrum of steroid substrates. However, the presence of other specific enzymes for 17- and 20-oxido-reduction, cannot be ruled out by these data. In this regard, 17ß-hydroxysteroid dehydrogenase has been cloned and shown to be different from 11ß-HSD (12). The binding of synthetic steroids (dexamethasone, R5020, and R1881) to 11ß-HSD is of interest, since these steroids are considered to be resistant

to metabolism. However, whether they are actually metabolized by 11ß-HSD remains to be determined.

Several implications can be drawn from the lack of specificity of 11ß-HSD. 11ß-HSD may contribute not only to cortisol resistance as we have recently suggested (1) but also to the resistance to progesterone and testosterone that also occurs in the squirrel monkey (2). The availability of cortisol to glucocorticoid receptors may be regulated by 11ß-HSD at three levels: 1) alterations in the enzyme concentration (3), 2) changes in the intracellular NADP/NADPH ratio (1), and 3) the presence of other steroids that are substrates or competitors of the enzyme. The latter would be important for steroids which normally are present at high concentrations such as dehydroepiandrosterone (DHEA) or progesterone during pregnancy. It is tempting to speculate that the Cushingoid appearance of male squirrel monkeys that appears during the breeding season (13) may be due to the large seasonal rise in testosterone levels. High levels of testosterone may inhibit 11ß-dehydrogenase in peripheral tissues resulting in increased cortisol availability to glucocorticoid receptors. Alternatively, the even higher levels of androstenedione (14) may reduce cortisol levels in feedback centers of the brain or pituitary by blocking cortisone conversion to cortisol thus increasing ACTH secretion. All of these possibilities depend upon the redox state of cellular pyridine nucleotide pools which in turn reflect the metabolic state of cells. Obviously these considerations may be extended to normal states. Indeed, 11ß-HSD may provide the long sought link between the hypothalamic-pituitary-adrenal axis and food intake.

The following lines of evidence strongly suggest that the binding sites identified in Sm cells are indeed 11ß-HSD: 1) the abundance of these binding sites

(55 pmol/mg protein) in Sm cells but not in Hu cells, 2) the higher affinity for cortisone (and cortisol) than for dexamethasone or triamcinolone, 3) the similar potency of several steroids for inhibition of [$^{3}$H]cortisone binding and 11ß-HSD activity, 4) affinity labeling of a 34 KDa band which is the reported molecular weight of 11ß-HSD purified from rat liver (15, 16) and 5) labeling of the 34 KDa band by [$^{3}$H]DM is blocked by cortisone and other subtrates of 11ß-HSD. These findings support our conclusion that increased 11ß-HSD activity in Sm cells is due to over expression of a relatively normal protein (1). Since 11ß-HSD is present in at least 100 fold higher concentration than glucocorticoid or other steroid receptors, receptor binding studies in Sm cells or other cells overexpressing this enzyme should be interpreted with caution.(5,7) Finally, the fact that 11ß-HSD binds cortisone with relatively high affinity can be exploited in studying this enzyme at the molecular level.

**MATERIALS**

Dulbecco's modified Eagle's medium (DME) (4.5g/L glucose, 0.584g/l L-Glutamine, 3.7g/L NaHC03) free of phenol; fungizone 250µg/ml; penicillin G 10,000 units/ml and streptomycin sulfate 10,000 mcg/ml; insulin 1mg/ml (24u/mg); and calf serum were obtained from the tissue culture facility of University of California, San Francisco. Six well plates were obtained from Becton Dickinson Labware, Lincoln Park, New Jersey. [1,2 $^3$H]cortisone was obtained from Amersham, Arlington Heights, Illinois. [1,2,6,7 $^3$H]Cortisol, [1,2,6,7 $^3$H]progesterone, [1,2 $^3$H]testosterone, 1ß [$^3$H]- androstenedione and [6,7 $^3$H]deamethasone mesylate were obtained from New England Nuclear Boston, MA; nonradioactive steroids from Steraloids Inc., Wilton, NH. Cell lines were obtained from the tissue culture facility of University of California, San Francisco, CA. Thin layer chromatography plates coated with silica gel were obtained from Whatman Ltd. Kent, England. Leupeptin, Aprotinin, Phenylmethylsulfonyl Fluoride (PMSF), Triton X100, DL-Dithiothretiol (DTT), Tris-HCl, glycerol, ethylene diamine tetraacetic acid (EDTA) and glycin were obtained from Sigma, St Louis, MO.

Acrylamide, N, N-methylene-bis-acrylamide and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad, Richmond, CA.

## METHODS

Cells were maintained in DME (phenol free) medium supplemented with 10% calf serum (CS), penicillin (100 units/ml) and streptomycin (100μg/ml), amphotericin β (Fungizone; 2.5μg/ml) and fibroblast growth factor (2ng/ml). Cells to be used in experiments, were plated in 6-well plates and fed with medium until they became confluent.

Tritium labelled cortisol, cortisone, progesterone, testosterone and androstenedione (specific activities of 99.8, 29, 109.3, 55.2 25.4ci/mmol respectively) were purified by celite column chromatography prior to use. The solvent systems were (by volume) iso-octane: t-butanol: $H_2O$ (250: 125: 225) for cortisol and (100: 35: 90) for cortisone; and iso-octane: t-butanol: methanol: $H_2O$ (110:10:70:10) for progesterone, and (50:20:20:10) for testosterone and androstenedione. The eluted peaks were collected, dried and reconstituted in ethanol and kept at $4^{o}$. After purification [$^3$H]- cortisone and [$^3$H]androstenedione contained less than 1% cortisol or testosterone whereas [$^3$H]cortisol, [$^3$H]progesterone and [$^3$H]- testosterone contained less than 2% cortisone, 20α reduced progesterone, or androstenedione as determined by thin layer chromatography (TLC). The data points were corrected for the amount of cross contamination determined in each experiment.

Stock solutions of steroids prepared in ethanol, and stored at $4^{o}$, were evaporated under a stream of nitrogen and reconstituted in DME before being added to cells.

Enzyme assays: monolayers of cells in 6-well plates were incubated with radioactive substrates in DME at 37° ($CO_2$ 5%, air 95%) for the indicated times. An aliquot of the medium was then extracted with 10 volumes of ethyl acetate. The ethyl acetate extract was evaporated under a stream of nitrogen and the radioactivity was reconstituted with 100μl ethyl acetate containing steroid standards (1mg/ml). Duplicate aliquots were applied to TLC plates and another aliquot was counted to monitor recovery. TLC plates were developed in chloroform: ethanol: t-butanol (9:1:1) to separate cortisol from cortisone or in chloroform: methanol (98:2.5) to separate progesterone from 20α reduced progesterone and testosterone from androstenedione. Following development, TLC plates were dried and the standard spots visualized by first spraying with a mixture of glacial acetic acid, $H_2SO_4$(conc.) and p-anisaldehyde (50:1:1) and then heating in a vacuum oven at 100° for 5-10 minutes. The identified steroid spots were cut out, eluted with 0.3ml of ethanol into mini-vials and the tritium counted after adding scintillation fluid. In preliminary experiments it was found that the spots containing the original steroid and its reduced or oxidized metabolite accounted for more than 95% of the total radioactivity recovered from the plates. Negligible radioactivity was associated with 20α– or 20β- reduced cortisone standards which were clearly separated from the cortisol standard. The recovery of the total radioactivity applied to the TLC plates averaged 80% and did not vary significantly between the controls (no cells) and the experimental points. Cell layers, washed twice with DME, were solubilized by incubating overnight with 0.5N NaOH at room temperature and cellular protein (about 300μg per well) was determined by the Bradford assay (17). Results are expressed as pmol/mg protein/hour of the product formed and were calculated by multiplying the percentage of substrate converted by the initial amount of substrate and then adjusting for protein and time of incubation. For kinetic studies, the data were linearized according to Eadie-Scatchard analysis ($V/S=V_{max}/K_m-V/K_m$,

where V is the rate in pm/mg/h and S is the substrate concentration in nM). A least squares best fit computer program was used to calculate the km and Vmax values. Inhibition experiments of 11ß-HSD activity were performed by the simultaneous addition to the cells of substrate and inhibitor desolved in DME and then the enzyme activity was determined as described above.

Whole cell binding assays: Confluent cells in six-wells plates were washed three times with DME to remove serum , preincubated for 1h at 4°, and then incubated with [$^3$H]cortisone with or without unlabelled steroids for 4h at 4°. After taking 200µl aliquots of the incubation medium to determine free [$^3$H]cortisone concentrations, cells were washed three times with cold DME (approximately two seconds each) and the radio-labelled hormones were extracted by incubation with 1ml methanol for 1h and counted after adding scintillation fluid. Scatchard analysis was performed on total cortisone bound and free values by computer assisted determination of non-specific binding .

Affinity labeling of cellular protein by [$^3$H]dexamethasone mesylate [$^3$H]DM): Confluent cells in 175 cM$^2$ flasks were washed twice with DME, harvested by genlty scraping with a rubber policeman, and collected by low speed centrifugation. Aliquots of resuspended cells were incubated with 100 pmol of [$^3$H]DM (specific activity 49.9 ci/mmol) ± 100 nmol of unlabeled steroids in 0.5 ml DME at 37° for 1h. Cells were then pelleted at 2000g at 4°, the supernatant carefully removed and the pellets immediately frozen at -70° for 15 min. Frozen pellets were resuspended in an equal volume of ice-cold lysing buffer (25mM Tris, pH 7.4, 10% glycerol, 1mM EDTA, 0.5µg/ml Leupeptin, 1µg/ml aprotinin, 0.2mM PMSF, 2mM DTT and 1% TRITON X100) thawed on ice, and centrifuged at 15000g at 4° to separate the lysate from cell debris.

Flurography: 5μl of cell lysate (about 20μg protein) were added to 25 μl loading buffer and immediately loaded on 10% (w/v) polyacrylamide denaturing gel overlaid by a 1 cm stacking gel and electrophorised according to the procedure of Laemmli (18). After gel electrophoresis, proteins were transferred electrophoretically (40 Volt, overnight, 4°) to a polyvinylidene difluoride membrane in a Bio-Rad mini protean II electroblotter. Loading buffer consisted of 62.5mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS w/v, 5% ß-mercaptoethanol V/V, 0.1% bromophenol blue w/v. Transfer buffer contained 0.025M Tris-HCl pH 8.3; 0.192M glycine, 20% methanol. After transfer the membrane was dried and sprayed with $EN^3HANCE$ spray before exposure to X-ray film. The fluorograph was scanned using a laser scan (Biomed Instruments). Bands were identified by their mobility and their densities were determined. The density of the band in the lane corresponding to cells incubated with [$^3$H]-DM alone was given an arbitrary value of 100.

Molecular weights were estimated according to the method of Weber and Osborn (19).

# TABLE 1

Inhibition of 11-oxo-reduction of [$^{3}$H]cortisone and 11ß- oxidation(11ß-dehydrogenase) of [$^{3}$H]cortisol by other steroids[a].

| | Fold excess | | | | |
|---|---|---|---|---|---|
| | X0.1 | X1 | X10 | X100 | X1000 |
| a) 11-oxo-reductase[b] | | | | | |
| 11 oxy-progesterone | 101 | 93 | 32 | 5 | 0 |
| cortisone | - | 109 | 72 | 19 | 0 |
| progesterone | - | - | 80 | 35 | 17 |
| 11ß-OH-progesterone | 106 | 102 | 96 | 49 | 20 |
| testosterone | - | - | 100 | 82 | 22 |
| cortisol | 94 | 99 | 101 | 104 | 86 |
| Ru486 | 92 | 93 | 97 | 98 | 93 |
| dexamethasone | - | 115 | 112 | 99 | 108 |
| b) 11ß-dehydrogenase[b] | | | | | |
| 11ß-OH-progesterone | - | 49 | 10 | 0 | 0 |
| cortisol | - | 63 | 23 | 7 | 0 |
| progesterone | - | - | 53 | 37 | 22 |
| testosterone | - | - | 74 | 35 | 20 |
| dexamethasone | - | - | 90 | 74 | 58 |
| cortisone | 120 | 110 | 178 | 272 | 228 |
| 11 oxo-progesterone | - | 62 | 176 | 181 | 109 |

| Ru486 | - | 71 | 85 | 103 | 171 |
|---|---|---|---|---|---|

a Data from one of two experiments with essentially identical results.

b Sm 5La cells were incubated for 10 minutes with 40nM [$^3$H]cortisone or [$^3$H]cortisol ± unlabeled steroids at the indicated fold excess. Results are the mean of duplicate assays (errors are typically less than 3%) and are expressed as percent of activities measured without inhibitors (145 and 16 pmol/mg/h for 11-oxo-reductase and 11ß-dehydrogenase, respectively).

# TABLE 2

[$^{3}$H]Cortisone binding to Sm cells.

| Number of washes | Total binding | Nonspecific binding | Specific binding |
|---|---|---|---|
| 0 | 110,294 | 54,563 | 55,731 |
| 1 | 92,478 | 18,882 | 73,595 |
| 2 | 75,776 | 11,995 | 63,781 |
| 3 | 72,302 | 8,519 | 63,783 |

Sm cells were washed three times with DME, preincubated at 4° for 1 h then incubated with 150 nM [$^{3}$H]cortisone ±30 μM unlabeled cortisone for 4 hours. Cells were then washed with 4° DME (each wash for two seconds). Results are expressed as cpm/well and are the average of triplicate values. Variability was less than 5% of mean value. Specific binding is the difference in cpm between samples with and without 200 fold excess unlabeled cortisone.

# TABLE 3

Inhibition of [$^3$H]cortisone binding to Sm cells.

| | Fold excess | | | |
|---|---|---|---|---|
| | 0.2 | 2 | 2 | 200 |
| 11 oxy-progesterone | 81 | 53 | 160 | 0 |
| 11ß-hydroxyprogesterone | 67 | 49 | 28 | 7 |
| cortisone | 100 | 66 | 17 | 0 |
| progesterone | 70 | 45 | 13 | 0 |
| cortisol | 103 | 77 | 24 | 17 |
| testosterone | 97 | 71 | 26 | 5 |
| aldosterone | 102 | 74 | 33 | 11 |
| androstenedione | 104 | 75 | 33 | 6 |
| 20α-hydroxy-pregn-4ene-3one | 93 | 90 | 52 | 5 |
| dihydrotestosterone | 113 | 92 | 56 | 27 |
| dexamethasone | 100 | 95 | 79 | 26 |
| triamcinolone | 100 | 102 | 97 | 71 |
| Ru486 | 103 | 92 | 101 | 82 |

The assays were performed as described in the Legend of table 2. Results are the mean of duplicate assays and are expressed as percent of specific binding. Specific binding was determined in the presence of 200 fold excess unlabeled

cortisone. contorl specific and non specific binding values were 82,000 and 10,600 cpm respectively.

-

Fig. 1

[$^3$H]cortisone binding to Sm cells at 4°. The assays were carried out as described in the legend of table 2. Kd = 446 ± 34nM, total binding = 68 ± 4.3pmol/mg protein.

Fig. 2

Affinity labeling of 11ß-HSD by [$^3$H]dexamethasone mesylate. Sm cells were incubated with 200nM $^3$H-DM ± unlabeled steroids for 1h at 37°. Cellular proteins were prepared by freeze-thaw lysis and analyzed by fluorography as described in methods. Standard proteins are myosin (H-chain) (200,000); phosphorylase b (97,000); bovine serum albumin (68,000); ovalbumin (43,000); carbonic anhydrase (28,000); ß-lactoglobulin (18,000, and lysozyme (15,000). 1, 11 oxyprogesterone; 2, estradiol; 3, triamcinolone; 4, progesterone; 5, testosterone; 6, cortisone; 7, dexamethasone; and 8, dexamethasone mesylate alone. Numbers under each lane represent relative density of the 34 KDa band obtained by densitometry (as described in methods), giving lane 8 with [$^3$H]DM alone an arbitrary value of 100. Results are typical of 3 experiments. Variation is ± 15%

Fig. 3

Inhibition of reduction of androstenedione to testosterone (a) and progesterone to 20α-hydroxy-pregn-4ene-3one (b) by 20 fold excess progesterone (p), androstenedione (A), cortisone (E), and triamcinolone (T). Sm cells were incubated at 37$^{o}$ with 660nM [$^{3}$H]androstenedione or 540 nm [$^{3}$H]progesterone ± competitors. Bars represent the mean of duplicate assays. The results are typical of two experiments.

Fig. 4

Inhibition of [$^{3}$H]androstenedione binding in Sm cells by nonradioactive progesterone (P), androstenedione (A), cortisone (E), and triamcinolone (T). Sm cells were incubated at 4° with 670 nM [$^{3}$H]-androstenedione ± unlabeled steroids for 4 h. Total binding was determined as described in the legend of Table 2. Values are the mean of duplicate assays. The results are typical of two experiments.

REFERENCES

1. Hammami, M.M., and Siiteri, P.K. 11ß-hydroxy steroid dehydrogenase (11ß-HSD) and cortisol resistance in the squirrel monkey. Submitted.

2. Coe, C.L., Smith, E.R., and Levine, S. (1985). The endocrine system of the squirrel monkey. In: Handbook of Squirrel Monkey Research. Rosenblum LA and Coe CL (eds), Plenum Press, NY, pp191-218.

3. Hammami, M.M., and Siiteri, P.K. Regulation of 11ß-hydroxy steroid dehydrogenase (11-HSD) activity in human skin fibroblasts; enzymatic modulation of glucocorticoid action. Submitted.

4. Pugeat, M.M., Chrousos, G.P., Nisula, B.C., Loriaux, D.L., Brandon, D., and Lipsett, M.B. (1984). Plasma cortisol transport and primate evolution. Endocrinology 115,357-361.

5. Chrousos, G.P., Loriaux, D.L., Brandon, D., Tomita, M., Vingerhoeds, A.C.M., Merriam, G.R., Johnson, E.O., and Lipsett, M.B. (1983). Primary cortisol resistance: A familial syndrome and an animal model. J. Steroid Biochem. 19,567-575.

6. Iida, S., Gomi, M., Moriwaki, K., Itoh, Y., Hirobe, K., Matsuzawa, Y., Katagiri, S., Yonezawa, T., and Tarui, S. (1985). Primary cortisol resistance accompanied by a reduction in glucocorticoid receptors in two members of the same family. J. Clin. Endocrinol. Metab. 60:967-971.

7. Chrousos, G.P., Renquist, D., Brandon, D., Eil, C., Pugeat, M., Vigersky, R., Cutler, J.R.,G.B., Loriaux, P.L., and Lipsett, M.B. (1982). Glucocorticoid hormone resistance during primate evolution: Receptor-mediated mechanisms. Proc. Natl. Acad. Sci. USA 79:2036-2040.

8. Cassorla, F.G., Albertson, B.D., Chronsos, G.P., Booth, J.D., Renquist, D., Lipsett, M.B., and Loriaux, D.L. (1982). The mechanism of hypercortislemia in the squirrel monkey. Endocrinology 111, 448-451.

9. Lopez-Bernal, A., Flint, A.P.F., Anderson, A.B.M., Thurnbull, A.C. (1980). 11ß-Hydroxysteroid dehydrogenase (11ß-HSD) activity (E.C.1.1.1.146) in human placenta, and decidua. J Steroid Biochem 12:1081-1087.

10. Murphy, B.E.P. (1981). Specificity of human 11ß-hydroxysteroid dehydrogenase. J Steroid Biochem 14:807-809.

11. Baggia, S., Albrecht, E.D., and Pepe, G.J. (1990). Regulation of 11ß-Hydroxysteroid dehydrogenase activity in the Baboon placenta by estrogen. Endocrinology 126:2742-2748.

12. Agarawal, A.K., Monder, C., Eckestein, B., and White, P.C. (1989) Cloning and expression of rat cDNA encoding corticosteroid 11ß-dehydrogenase. J Biological Chemistry. 264(32):18939-18943.

13. Coe, L.C., Smith, E.R., and Levine, S. (1985). The endocrine system of the squirrel monkey In Handbook of squirrel monkey research. Rosenblum, L.A. and Coe, C.L. (eds.). Plenum Press, N.Y.

14. Siiteri, P.K. (1986). High plasma steroid levels in the squirrel monkey deficient receptors or metabolism? In Steroid Hormone Resistance. Chrousus, G.P., Loriaux, D.L., and Lipsett, M.B. (eds.). Plenum Press, N.Y.

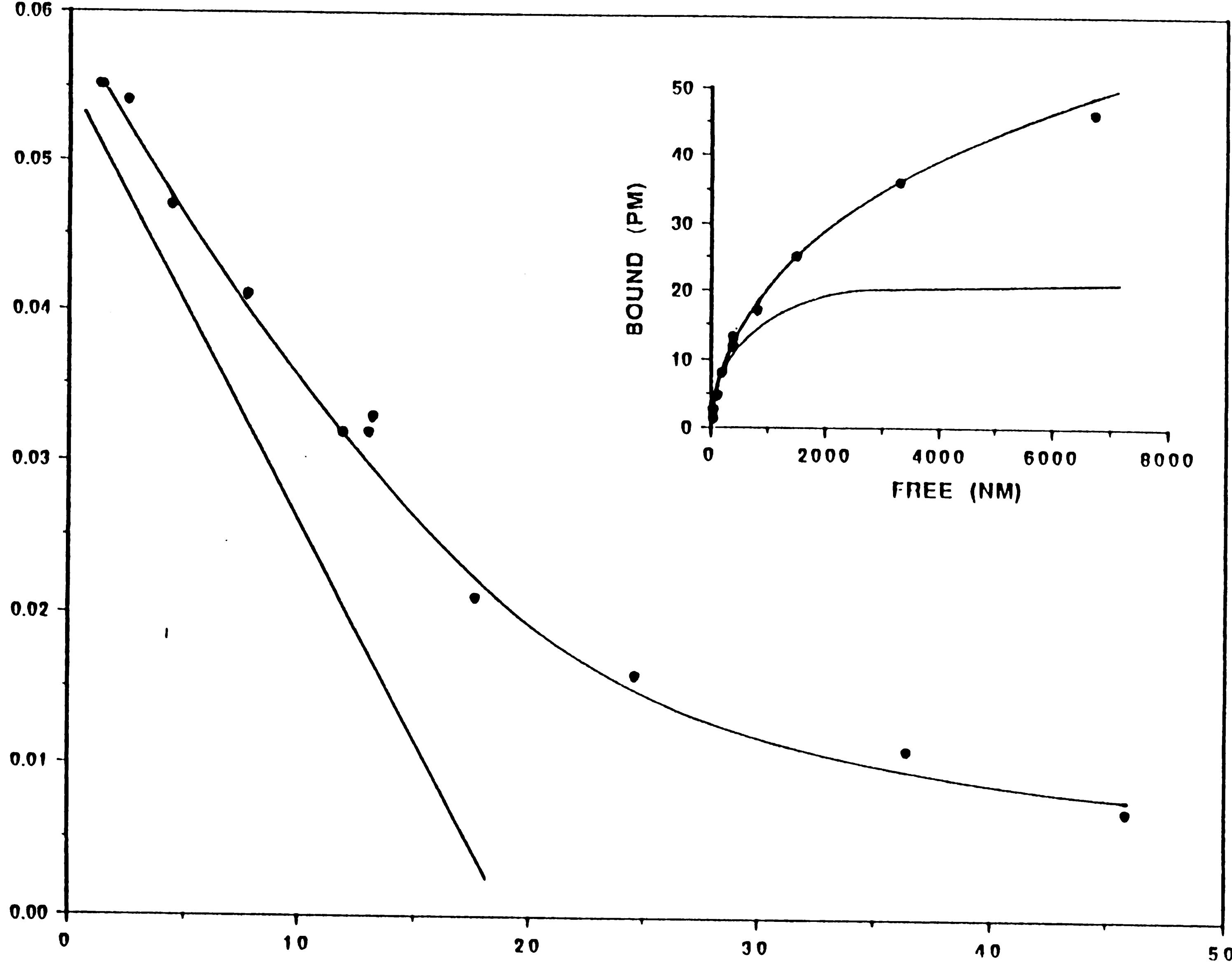
15. Lakshmi, V., and Monder, C. (1988) Purification and characterization of corticosteroid 11ß-dehydrogenase component of the rat liver 11ß-hydroxysteroid dehydrogenase complex. Endocrinology 123:2390-2398.

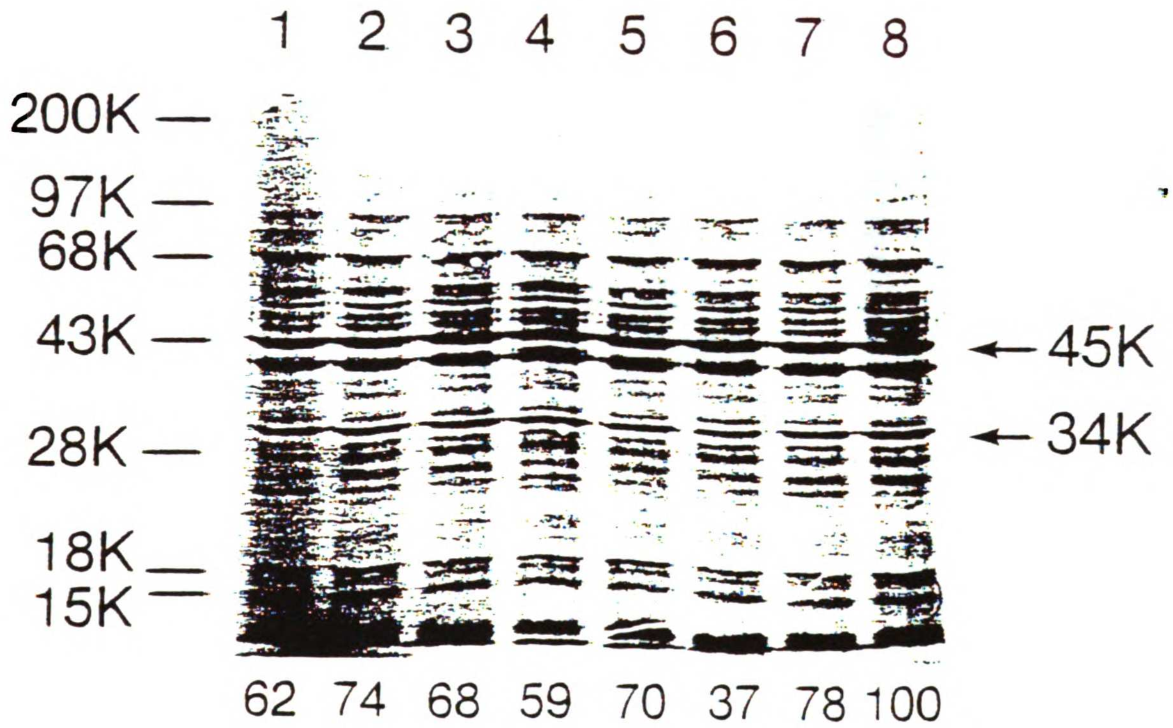
16. Monder, C., and Lakshmi, V. (1990) Corticosteroid 11ß-dehydrogenase of rat tissues : Immunological studies. Endocrinology. 126:2435-2443.

17. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochem. 72:248-254.

18 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature 227:680-685.

19. Weber, K., and Osborn, M. (1969) The reliability of Molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J Biological Chemistry 244(16): 4406-4412.


BOUND/FREE
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
BOUND (PM)
50
40
30
20
10
0
0
2000
4000
6000
8000
FREE (NM)


1
2
3
4
5
6
7
8
200K
97K
68K
43K
28K
18K
15K
45K
34K
62
74
68
59
70
37
78
100

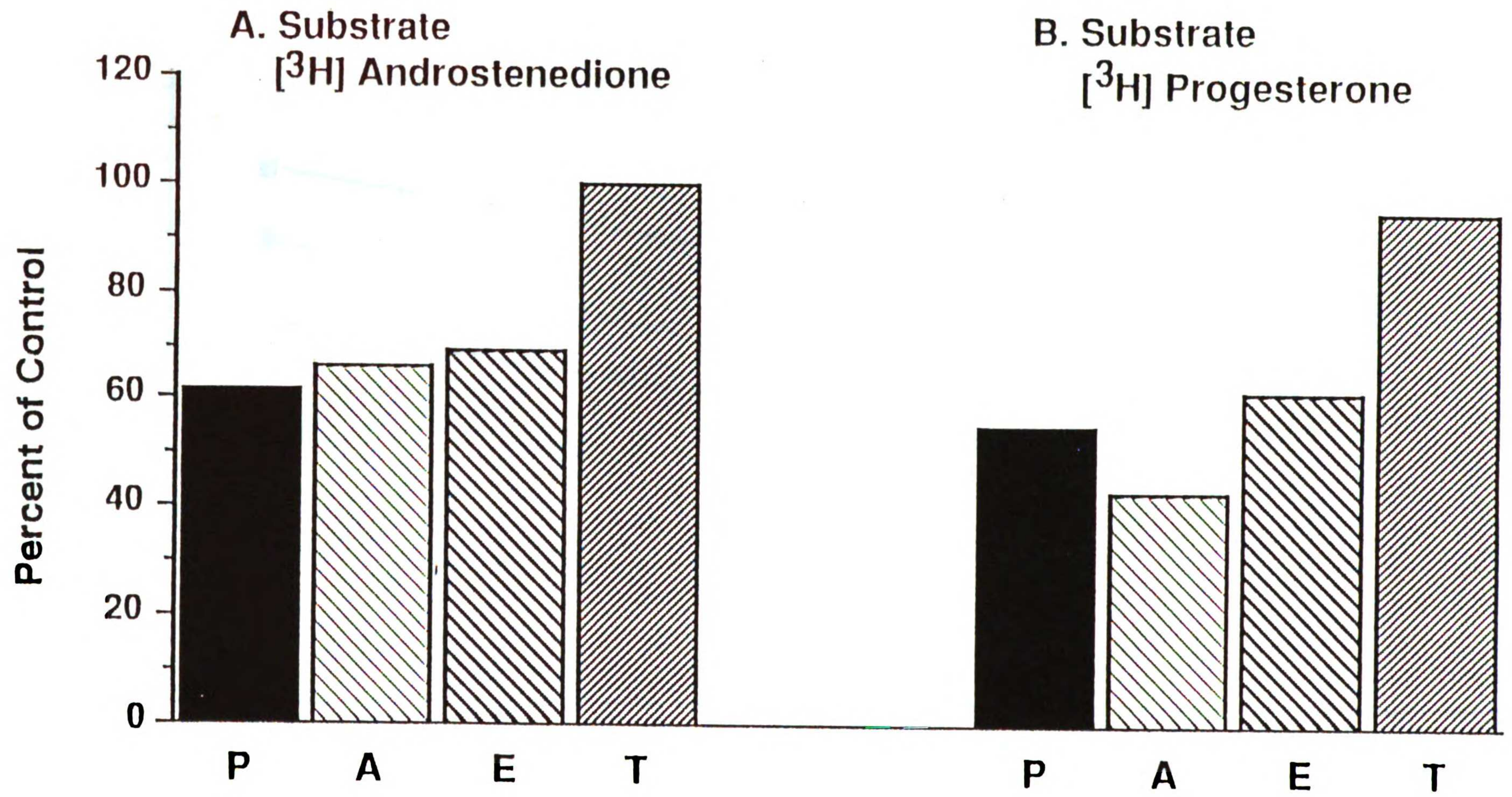



Fig. 3

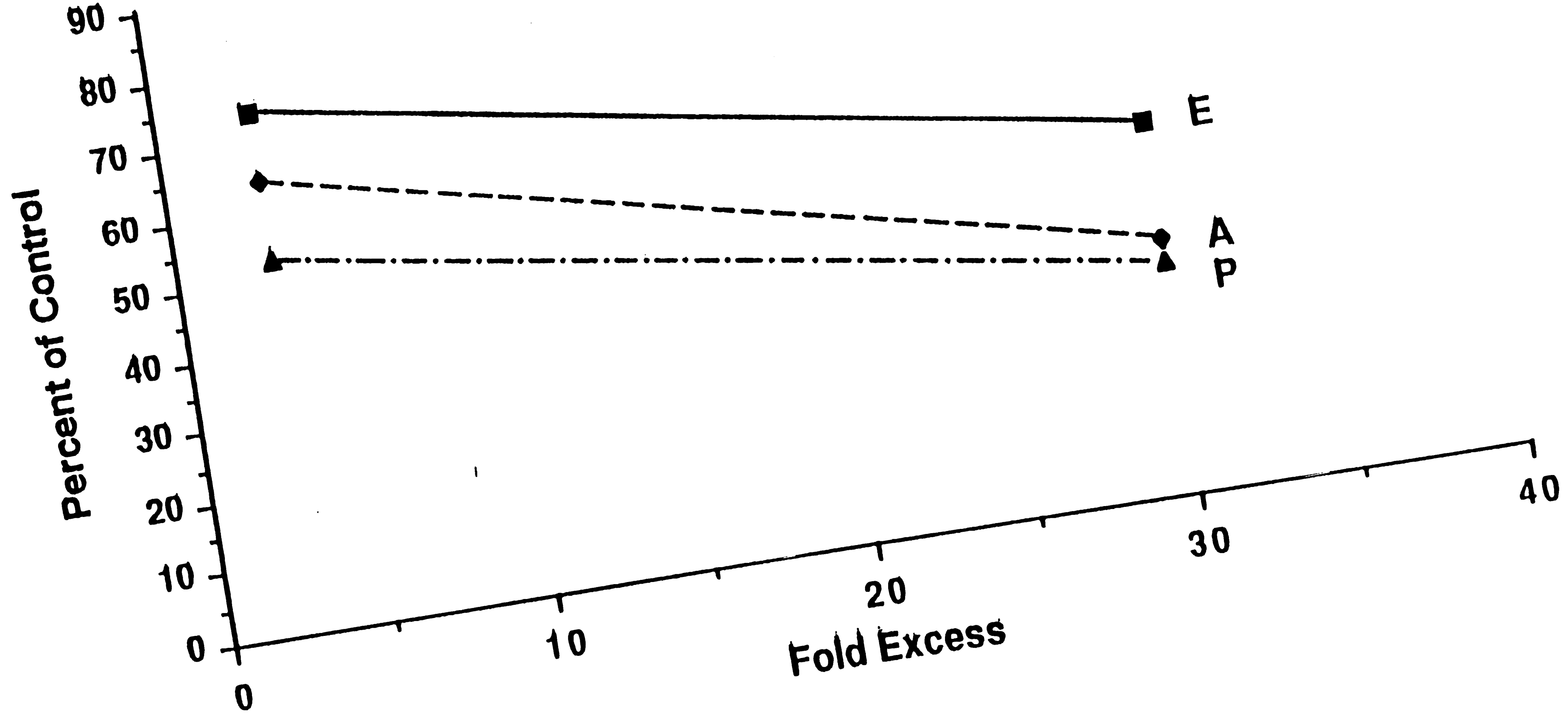



Fig. 4

## SUMMARY AND CONCLUSIONS

1. 11ß-Hydroxy Steroid Dehydrogenase (11ß-HSD) in human skin fibroblasts.

a) 11-Oxo-reductase activity (cortisone to cortisol) in human skin fibroblasts is 5-10 fold higher than 11ß-dehydrogenase activity (cortisol to cortisone). This is due to 5-10 fold higher Vmax (20 vs. 2-4 pmol/mg protein/h) and similar Km (220 nM).

Since the effect of steroid hormone is directly related to their receptor occupancy and the level of free cortisol is normally around the Kd for glucocorticoid receptors (GCR), any change in cortisol level results in a proportional change in GC activity.

How significant is the contribution of 11ß-HSD to the availability of cortisol to GCR? In a simplistic view is can be calculated that 11ß-HSD could potentially metabolize 50 percent of the cortisol produced in 24 h [given a Vmax of 10 mol/mg protein/h (16,000 µm/70 Kg/24 h), a Km of 200 nM, free cortisol concentration of 20 nM, and a 24 h production rate of 10 mg (3 m moles)]. Further, only a small fraction of the total cortisol produced escapes metabolism by the liver (as reflected by the ratio of urinary free cortisol to 17 hydroxycorticosteroid); thus peripheral 11ß-HSD has the potential of metabolizing much more that 50 percent of the cortisol otherwise available to GC receptors. To experimentally quantitate the effect of 11ß-HSD on cortisol availability to GCR, one can determine the effectivenss of cortisol (and cortisone) in competing with [$^{3}$H]dexamethasome for GCR in the presence or absence of 11ß-HSD inhibitors. Similarly the effectiveness of cortisol (and cortisone) in inducing aromatase activity can be determined.

b) Glucocorticoids induce 11ß-HSD activity three fold. Other steroid hormones (testosterone, dehydrotestosterone, progesterone, estradiol and triiodothyronin) are ineffective. The induction with GC is likely via GCR since 1) dexamethasone and cortisol induce 11ß-HSD activity half maximally at concentrations that parallel their Kd for GC R, 2) GCR antagonists prevent the induction by GC, and 3) the induction requires mRNA and protein synthesis. This induction is delayed (half maximal at 48 h) suggesting a secondary rather than a primary effect.

Such secondary effect may be via an increase in the level of a stimulatory intermediate(s) or a reduction in the level of an inhibitory intermediate(s). The later could be GCR itself. In this event the induction of the synthesis of 11ß-HSD by GC may be a disinhibition effect due to down regulation of GCR. This may explain the ineffectiveness of GC in inducing 11ß-HSD in squirrel monkey fibroblasts (see 2d). Given the arguments in 1a), the three fold increase in 11ß-HSD Vmax by GC would be of prime physiologic significance. Depending on the redox state of the cell (the ratio of NADP/NADPH) GC activity will either decrease or increase as compared with base line. Thus the induction of 11ß-HSD by GC could be considered a compensation mechanism for down regulated GCR in some tissues (where oxo-reductase>> dehydrogenase) or, along with GCR down regulation, a protection mechanism against excessive GC activity in other tissues (where dehydrogenase>> oxo-reductase). In this regard, it is of major interest to study 11ß-dehydrogenase and 11ß-oxo-reductase activities and the regulation of 11ß-HSD in other GC target tissues.

c) Serum factor(s) suppress(es) 11ß-HSD activity. This suppressive activity is present in all four sera examined: human, calf, squirrel monkey and rhesus monkey. Treatment of serum with charcoal did not decrease the suppressive activity. The increase in 11ß-HSD activity upon serum removal was seen as early as 18 h and continued to increase to six fold in five days. This increase in activity

is due to increased synthesis of 11ß-HSD protein as reflected by 1) increase in Vmax without a change in Km, 2) dependency on protein and mRNA synthesis. Insulin was identified as suppressor of 11ß-HSD activity induced by serum removal or by GC treatment. Although insulin is able to suppress 11ß-HSD activity to levels similar to those observed in cells grown in 10 percent calf or human serum wtih EC50 of 1-5 nM, it is likely that there are other suppressive serum factors since there is only 0.1-0.5 nM insulin in medium containing 10 percent calf serum. EGF and FGF were less effective than insulin. However, $IGF_1$, $IGF_2$ and other growth factors were not examined. Other serum factors that have cAMP or PKC in their signaling pathway are likely to be responsible since cAMP and/or PMA partially suppressed 11ß-HSD activity. These factors need to be identified. Further, that GC induces and insulin suppresses the synthesis of 11ß-HSD mRNA and 11ß-HSD protein needs to be confirmed by Northern blots and Western blots/autoradiography analysis.

d) 11ß-Dehydrogenase and 11ß-oxo-reductase activities (and Vmax) are modulated by several effectors in parallel. This proves that the changes in activities are not due to altered redox state of cells and strongly suggests that one protein catalyzes both activities.

As detailed in literature review, the common notion was that there are two distinct proteins that are capable of either 11ß-oxidation (dehydrogenase activity) or 11ß-reduction(oxo-reductase activity). However, recently rat liver 11ß-HSD cDNA was cloned and the expressed protein had both activities. Given this new information, studies which used the presence of 11ß-HSD protein (as reflected by immunohistochemistry or Western blotting) as an indicator of cortisol inactivation to cortisone should be interpreted with caution. Further, even determining 11ß-dehydrogenase activity is not enough since net inactivation of cortisol depends on

the ratio of 11ß-dehydrogenase to 11-oxo-reductase activities. Thus, both activities should always be determined.

How can one account for the following observations noted in earlier studies?: 1) Wide variability of the ratio of 11ß-dehydrogenase to 11-oxo-reductase activitiesin different tissues, 2) different physiochemical properties of 11ß-dehydrogenase and 11-oxo-reductase activities, and 3) clinical examples of patients with 11ß-dehyerogenase activity without 11-oxo-reductase activity and vice versa. It is conceivable that all these observations are due to different cofactors ratio (NADP/NADPH) in different cells and/or to the stability of a specific cofactor binding site on the enzyme. A mutation or posttranslational modification of these sites may result in a unidirectional enzyme. Thus, although one protein can catalayze both the oxidation and reduction reactions, there may exist proteins with only one activity. In this regard, it is interesting that our preliminary studies on 11ß-HSD activity in a human cancer cell line (T47D) has shown 11ß-dehydrogenase activity with a Km one order of magnitude lower than that determined in normal human fibroblasts. Further, no 11-oxo-reductase activity was found in these cells.

e) In human skin fibroblasts, apparent 11ß-dehydrogenase activity can be increased by adding cortisone simultaneously with the labeled substrate. As discussed in the manuscript, this is due to inhibition of the reductase activity by the added cortisone. Similarly, when 11ß-dehydrogenase is the predominant activity (at 4°C) adding cortisol with the labeled substrate increases apparent 11-oxo-reductase activity.

Failure to suppress one of the two reactions may result in a false conclusion that in some cells one activity (dehydrogenase or oxo-reductase) is present in the absence of the other. This is especially important when measuring activities in intact cells; in cell free preparation the addition of the appropriate cofactor only will prevent this pitfall.

f) Cortisone can be biologically active depending on local 11ß-HSD activity. 200 nM cortisone can induce aromatase activity seven fold after 18h incubation. Further, the bioactivity of cortisone increases up to 50% of that of cortisol when 11ß-HSD activity is induced by serum removal. Since 200 nM is only 2-3 fold of physiologic cortisone concentration this clearly shows the possible contribution of 11ß-HSD and cortisone to GC activity in vivo.

2. <u>11ß-HSD in Squirrel Monkey (SM) Skin Fibroblasts</u>.

a) 11ß-dehydrogenase activity is several hundred fold higher in SM cells as compared to rhesus monkey (Rm) and human (Hu) cells due to several hundred fold higher Vmax without a significant difference in Km suggesting overexpression of normal 11ß-HSD protein in Sm cells This was further confirmed by whole cell binding assays and protein labeling (see 2f) below). The increased activity was seen in all Sm cell lines examined and in none of the Hu or Rm cell lines. Further there was no difference between sexes, subspecies or different animals nor between peripheral, omental or genital skin fibroblasts. Furthermore, there was no difference between cell passages (up to 25). Although this strongly suggests that this increase in activity is present in all Sm cells, other cells/tissues need to be examined.

b) The ratio of 11ß-dehydrogenase to 11-oxo-reductase activities is 5-10 times higher in Sm cells as compared to Hu cells.

Net inactivation of cortisol depends on the ratio of 11ß-dehydrogenase to 11ß-oxo-reductase activities. Sm cells not only overexpress 11ß-HSD but also favor formation of cortisone. That overexpression of 11ß-HSD may be the cause of glucocorticoid resistance is in agreement with the proposed role of this enzyme in mineralocorticoid target tissues,the placenta,and the testis. Moreover,overexpressed 11ß-HSD could readily explain the absence of inadverent mineralocorticoid activity despite extremely high cortisol levels,and the apparent decrease in cortisol affinity for otherwise normal aldosterone receptors in squirrel monkeys. It is of prime interest to see if this enzyme is "the site of metabolism" that contributes to the apparent glucocorticoid resistance in the guinea pig , another new world animal..The observation that cortisol levels are resistant to suppression by dexamethasone is in conflict with this hypothesis,since dexamethasone appears to be a poor substrate for 11ß-HSD. However, this has not been unequivocaly

shown. Further,unsuppressed cortisol levels may be due to some degree of autonomy of SM adrenals rather than glucocorticoid resistance at the hypothalamic/pituitary level. ACTH levels need to be measured to clarify this point. In fact, despite similar levels of ACTH, cortisol production rate is 6 fold higher in SM as compared to cynomolgus monkey. Furthermore,dexamethasone which is known to inhibit 11ß-HSD, may prolong the already increased cortisol half life giving rise to false negative suppression test. Further in vivo studies are clearly needed.

c) The ratio of 11ß-dehydrogenase to 11-oxo-reductase activities can be changed by altering the intermediary metabolism of cells such by incubation in glucose free medium or at 4°C. This observation is likely due to a decrease in the formation of NADPH since it is glucose and temperature dependent. The ratio of 11ß-dehydrogenase to 11-oxo-reductase activities in vitro may or may not reflect that in vivo. If it is lower than predicted,11ß-HSD may increase rather than decrease GC availability. Hence, the determination of the ratio in vivo is of import. This can be accomplished by injecting [$^3$H] cortisol and [$^{14}$C] cortisone in the artery of an animal limb and measuring the ratio of [$^3$H] cortisol/[$^3$H] cortisone and [$^{14}$C] cortisone/[$^{14}$C] cortisol in the venous effluent of the same limb. This will avoid the contribution of the liver and/or the kidney. Although overexpression of 11ß-HSD in Sm cells may be a cause for GC resistance, other defect(s), such as a decrease in GCR numbers and/or affinity or post-receptor defects(s) may also contribute to GC resistance in this species. To prove the causality of 11ß-HSD to GCR and to quantitate its role, it is necessary to show a decrease in cortisol levels by inhibiting 11ß-HSD while maintaining normal health. Further, overexpression of 11ß-HSD cDNA in normal cells or in a transgenic mice should result in GC resistance.

d) 11ß-HSD in squirrel monkey cells is not regulated by glucocorticoid, insulin or serum removal.

Although human insulin ineffectiveness in squirrel monkeys could be a general phenomenon that is not specific to 11ß-HSD, the lack of effect of GC appears to be specific. As discussed above, the increase of 11ß-HSD by GC in human cells may be a disinhibition rather than true induction. In this case, 11ß-HSD in Sm cells may be constitutively overexpressed and thus cannot be disinhibited. It will be of great interest to clone 11ß-HSD gene from Sm cells to compare its 5-end (and other possible regulatory sequences) with those of human and rat 11ß-HSD genes. It is expected that a mutation in some trans-acting sequences would be identified. This may lead to identification of negative cis-acting factors. Alternatively a defect in cis-acting factor(s) may be the underlying abnormality.

e) 11ß-HSD in squirrel monkey cells has a broad substrate specificity. It was shown that testosterone and progesterone among other steroids competitively inhibit the interconversion of cortisol and cortisone. Further, testosterone and progesterone are converted to their corresponding reduced or oxidized counterparts by Sm cells. Furthermore, this conversion is inhibited by each other and by cortisone. However, this non-specificity is not absolute, since cells interconverted cortisol and cortisone ten times more rapidly than testosterone and androstenedione or progesterone and 20 $\alpha$-hydroxyprogesterone.

The nonspecificity of 11ß-HSD suggests a role of its overexpression in Sm cells in generalized steroid hormones resistance known to occur in squirrel monkeys. Alternatively, other steroid hormones can modulate GC availability to GC receptors by altering 11ß-HSD activity when serving as substrates or inhibitors. The ineffectiveness of the synthetic steroids in inhibiting 11ß-HSD explains their prolonged biologic half life, and advantageous feature. However, thought should be given to their resistance to local fine tuning by 11ß-HSD in

individual tissues. It remains to be shown that similar GC effectiveness of cortisol and dexamethasone can be achieved in vivo with fewer side effects with the former. f) Cortisone binds to 11ß-HSD with high affinity (Kd = 400 nm). This binding permitted the following: 1) quantitation of the enzyme in intact cells, 2) characterization of the inhibition of 11ß-HSD by other steroid as competitive, and 3) affinity labeling of 11ß-HSD protein and studies at the molecular level.

Undoubtedly, the role of 11ß-HSD in steroid hormone action will be of increasing importance not dissimilar to the role of 5 α-reductase in androgen action and 5'deiodinase in thyroid hormone action. I hope this work is a step forward.

## VI. REFERENCES


1. Lakshmi ,V., and Monder,C.(1985) Endocrinology 116( 2), 552-560.
2. Berliner ,D.L., and Ruhmann,A.G.(1966) Endocrinology 78, 373 -382.
3. Murphy,B.E.B.,(1981) J. Steroid Biochem. 14, 811-817.
4. Weidenfeld,J., Siegal,R.A.,Levy,J., and Chowers,I.(1982) J. Steroid Biochem.17, 357-360.
5. Lakshmi ,V., and Monder,C.(1988), Endocrinology 123( 3)2390 -2398.
6. Abramovitz ,M.,Branch,C.L.,and Murphy,B.E.P.(1982) J. Clin. Endocrinol. Metab 24, 563-568.
7. Stewart,P.M.,Corrie,J.E.T.,Shackelton,C.H.L. and Edwards,C.R.W.(1988), J. Clin. Invest. 82,. 340 -349.
8. Taylor,N.F.,Bartlett,W.A.,Dawson,D.J.,Enoch,B.A.(1984) J. Endocrinology 102 (supp), p. 90
9. Phillipou,G., and Higgins,B.A.(1985)J. Steroid Biochem. 22,435-436.
10. Schulz ,W.,Lichtenstein,I.,Siebe,H.,and hierholzer,K(1989) J. Steroid. Biochem. 32,(4). 581-590.
11. Agarwal,A.K.,Monder,C.,Eckstein,B.,and White,P.C.(1989) J. Biol. Chemistry. 264(32),18939-18943.
12. Monder,C., and Lakshmi,V.(1989)J. Steroid Biochem.32,(1A)77-83.
13. Kabayashi,N.,Schulz,W.,and Hierholzer,K.(1987) Pflüger Arch. 408, p. 46-53.
14. Lopez-Bernal,A.,Flint,A.P.F.,Anderson,A.B.M.,and Turnbull,A.C.(1980) J. Steroid. Biochem.13, 1081-1087.
15. Lakshmi,V., and Monder,C.(1967) Endocrine Society Annual Meeting Abstract #402
16. Murphy,B.E.P.(1989) J. Steroid Biochem. 14, 811-817
17. Tye ,L.M. and Burton,A.F.(1980) Life Sciences 26, 35-39.

18. Monder,C.,and Shackleton,C.H.L.(1984) Steroids (1984).44, (5)383 - 417.

19. Dougherty ,T.F.,Berliner,M.L., and Berliner,D.L.(1960) Endocrinology 66,-558. 550

20. Smith ,B.T.,Torday,J.S. and Giround,C.J.P.(1973) Steroids 22,515-524.

21. Zumoff ,B.,Bradlaw,H.L.,and Levin,J.(1983) J. Steroid Biochem. 18, (4) 437-440.

22. Koerner,D.R., and Hellman,L.(1964) Endocrinology 75, 592-601.

23. Pepe,G.J.,Waddell,B.J.,Stahl,S.J.,andAlbrecht,E.D.(1988)Endocrinology 122,(1)78-83.

24. Abramovitz ,M.,Carriero,R., and Murphy,B.E.P.(1984) J. Steroid Biochem. 21, 677-683.

25. Funder,J.W.(1987)Science 237, 236-237

26. Edwards ,C.R.W.,Burt,D., and Stewart,P.M.(1989) J. Steroid Biochem. 32,( 1B) 213-216.

27. Edwards,C.R.W.,Stewart,P.M.,Burt,D.,Brett,L.,McIntyre,M.A., Sutanto,W.S.,Deloet,E.R.,and Monder,C.The Lancet(october 29,1988) 986-989.

28. Funder,J.W.(1989) Endocrine Research 15, 227-238.

29. Funder ,J.W.,Pearce,P.T.,Smith,R., and Smith,A.I.(1988) Science 242, 583-585.

30. Spieth,A., Hierholzer,K.,tech Asst. Lichtenstein,L., and Siebe,H.(1987) pFlüger Arch48,R42-47.

31. Rossier,B.C.(1989) Endocrine Research 15, 203-226.

32. Ulick,S.,Levine,L.S.,Gunczler,P.,Zunconato,G.,Romiverz,L.C., Ranh,W.Rosler,A.,Bradlow,H.L., and New,M.I.(1979) J. Clin. Endo. Metab. 49,(5) 757-764.

33. Ulick,S.,Chan,C.K., Rao,K.N.,Edassery,J.,Mantero,F.,(1989)J. Steroid Biochem.32,(1B)209-212.

34. Oberfield,S.E.,Lenore,S.L.,Carey,R.M.,Greig,F.,Ulick,S., and New,M.I.(1983)J. Clin. Endo. Metab.56,(2)332-339.

35 Monder,C.,Shackleton,C.H.L.,Bradlow,H.L.,New,M.I., Stoner,E.,Iohan,F., and Lakshmi,V.(1986)J. Clin. Endo. Metab.63,(3)550-557.

36. Arriza,J.L.,Weinberger,C.,Cereili,G.,Glaser,T.M.,Hendelin,B.L., Housman,D.E.,and Evans,R.M.(1987)Science 237, 268-275.

37. Epstein,M.T.,Espiner,E.A.,Donald,R.A.,Hughes,H.,Cowles,R.J., Lun,S.(1978)J. Clin. Endocrinol.. Metab. (1978)47,(2)397-400.

38. Stewart,P.M.,Wallace,A.M.,Valentino,R.,Burt,D.,Edwards,C.R.W. The Lancet (October 10, 1987),821-824

39. Monder,C.,Stewart,P.M.,Lakshmi,V.,Valentino,R.,Burt,D., Edwards,C.R.W.(1989)Endocrinology 125,(2)1046-1053.

40. Takeda,R.,Miyamori,I,Soma,R.,Matsubara,T. and Matsatoshi,I.(1987) J. Steroid Biochem.27,(4-6)845-849.

41. Hughes,R.M.(1977) N.Z. Med. J.85,398-400.

42. Stewart,P.M.,Valentino,R.,Edwards,C.R.W.(1988)Serono Symposium on the Adrenal Gland and Hypertension from cloning to clinic, Tokyo, Japan

43. Phillips ,D.M.,Lakshmi,V., and Monder,C.(1989) Endocrinology 125,(1)209-216.

44. Vingerhoebs,A.C.M.,Thijssen,J.H.H., and Schwarz,F.(1976)J. Clin. Endocrin. Metab.43,1128-1133.

45. Chrousos,G.P.,Vingerhoebs,A.C.M.,Loriaux,D.L., and Lipsett,M.B.(1983)J. Clin. Endocrin. Metab.56,(6)1243-1245.

46. Chrousos,G.P.,Vingerhoebs,A.C.M.,Brandon,D.,Eil,C.,Pugeat,M.,

Devrede,M.,Loriaux,D.L., and Lipsett,M.B.(1982)J. Clin. Invest. 69,1261-1269.

47. Lipsett,M.B.,Tomita,M.,Brandon,D.D.,Devroede,M.M.,Loriaux,D.L., and Chrousos,G.P.(1985) In advances in Exp. Medicine and Biology, Steroid Hormone Resistance and Clinical Aspects (Chrousos, L. & Lipsett, eds) vol. 196,97-109.

48. Carter,K.M.,Berkovitz,G.D.(1989) 71st Endocrine Society Meeting Abstract #659.

49. Iida,S.,Gomi,M.,Moriwaki,K.,Itoh,Y.,Hirobe,K.,Matsuzawa,Y.,Katagiri ,S.,Yonezawa,T., and Tarui,S.C.(1985)J. Clin. Endocrinol Metab.60,(5) 967-971.

50. Gomi,M.,Iida,S.,Tsugaua,M.,Itoh,Y.,Moriwaki,K.,Yamashita,S., and Tarui,S.(1986)New Engl. J. Med314,(18)1194

51. Iida et al, 71st Endocrine Society Meeting (1989), p. 98, Abstract #303

52. Danoff,A.,Jormark,S.,Lorber,D.,Fleischer,N.(1987)Arch. Intern. Med. 47,443-448.

53. Carney,J.A.,Gordon,H.,Carpentor,P.C.,Shenoy,B.V.,Go,V.L.W.(1985) Medicine 64,270-283.

54. Kontula,K.,Pelkonen,R.,Anderson,L.,and Sivula,A.(1980)J. Clin. Endocrinol. Metab. (1980)51,654-0657.

55. Nawata,T.M.(1984) 7th International Congress of Endocrinology, Quebec, Canada.Abstract #1605

56. Cohen,M.Stiefel,M.,Reddy,W.J.,Laidlow,J.C.(1958) J.Clin.Endocrinol.Metab.18,1076-1092.

57. Nolten,W.E.,Lindheimer,M.D.,Rueckert,P.A.,Oparil,S.,and Ehrlich,E.N.(1980)J. Clin. Endocrinol. Metab 51,466-472.

58. Rosenthal,H.,Slaunwhite,W.R.,Sandberg,A.A.(1969) J.Clin.Endocrinol.(1969)29,352-367.

59. Nolten,W.E.,Rueckert,P.A.(1981) Am. J. Obstet. Gynecol.139,492-498.

60. Menlenberg,P.M.M.,Ross,H.A.,Swinkels,L.M.J.W.and Bernard,T.J.(1987)Clinica Chimica Acta 165, 379-385.

61. Baulieu,J.O.and Chin,S.S.(1956)J. Clin. Endocrinol. Metab. 16,690-694.

62. Robertson,M.E.,Stiefel,M.,Laidlaw,J.C.(1959)J.Clin.Endocrinol.Metab. 19,1381-1396.

63. Peterson,R.E.,Nokes,G.,Chen,P.S.,Black,R.L.(1960)J.Clin.Endocrinol. Metab.20,495-514.

64. Plager,J.E.,Schmidt,K.G., and Staubitz,W.J.(1964)J.Clin. Invest.43,1066-1072.

65. Casper,R.C.,Chatterton,R.T. and Davis,J.M.(1979)J. Clin. Endocrinol. Metab.49,406-411.

66. Rolla,m.,D.,Chicca,M.G.,Andreoni,A.,Belliti,D.,Devescovis,S.,Andreani ,G.,and Nerica,A.(1984)J. Endocrinol. Invest. 17,243-247.

67. Boyar,R.M.,Hellman,L.D.,Roffworg,H.,Katz,J.,Zumoff,B.,O'Conner,J. Bradlow,H.L.,Fukushima,D.K.(1977)N.E.J.M 296,190-193.

68. Walsh,B.T.,Katz,J.L.,Levin,J.,Kream,J.,Fukushima,D.K.,Weiner,H., and Zumoff,B.(1981)J. Clin. Endocrinol. Metab. (1981),53,203-205.

69. Peterson,R.E.,(1958) J. Clin. Invest.37,736-743.

70. Konlula,K.,Anderson,L.C.,Huttumen,M.,and Pelkonen,R.(1982)Horm. Metab. Res 14,619-620.

71. Vierhopper,H.,Kiss,A.,Nowotny,P.,Wadhausl,W.(1989) 71st Endocrine Society Meeting Abstract #656

72. Chrousos,G.P.,Renquist,D.,Brandon,D.,Eil,C.,Pugeat,M., Vigersky,R.,Cutler,G.B.,Jr.,loriaux,D.L.,and Lipsett,M.B.(1987)

Proc. Nat. Acad. Sci. USA 79,2036 -2040.

73. Pugeat,M.M.,Chrousos,G.P.,Nosula,B.C.,Loriaux,D.C.,Brandon,D.,and Lipsett,M.B.(1984), Endocrinology.115,357-361.

74. Cassorla,F.C.,Albertson,B.D.,Chrousos,G.P.;Booth,J.D., Renquist,D.,and loriaux, D.L.(1982) Endocrinology 111,448-451.

75. Chrousos,G.P.,Loriaux,D.L.,Brandon,D.,Shul,J.,Renquist,D., hogan,W.,Tomita,M and Lipsett,M.B.(1984)Endocrinology 115,25-30.

76. Chrousos,G.P.,Loriaux,D.L.,Tomita,M.,Brandon,D.,renquist,D., Albertson,B., and Lipsett,M.B.In advances in Experimental Medicine and Biology.Steroid Hormone Resistance Mechanism and Clinical Aspects, (Chrousos, Loriaux and Lipsett, eds),196,129-144.

77. Hodgson,A., and Funder,J.W.(1978) Am. J. Physiol.235,(3)R115

78. Kraft ,N.,Hodgson,A.J., and Funder,J.W.(1979) Endocrinology 104,344-349.

79. Sibley,C.H. and Tomkins,G.M.(1974) Cell 2,221-227.

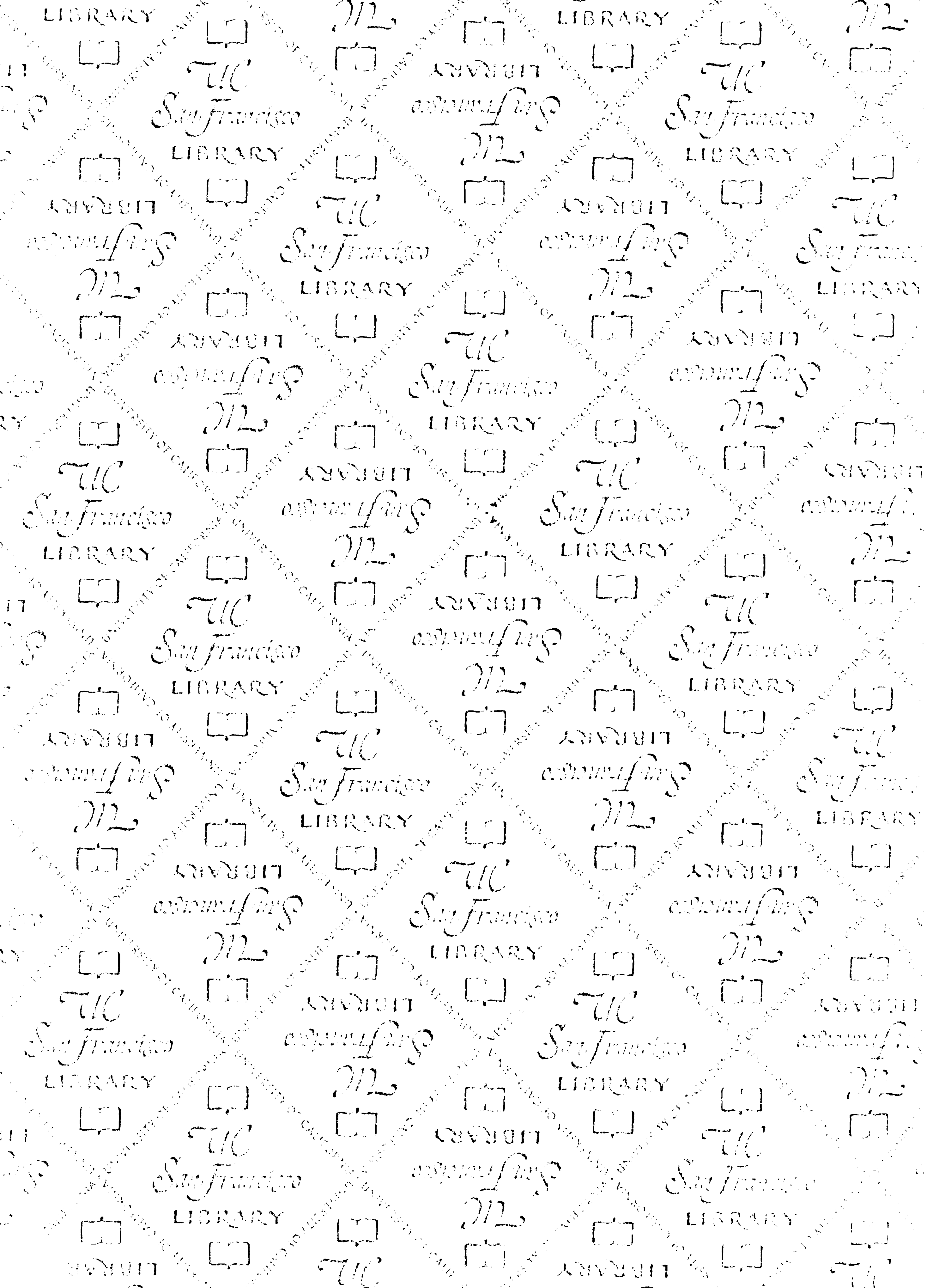
80. Thompson,B.,and Harman,J.M. In advances in Experimental Medicine and Biology. Steroid Hormone Resistance Mechanism and Clinical Aspects, (Chrousos, Loriaux and Lipsett, eds),196,111-127.

81. Berliner,D.L.,(1956) Cancer Research 25,1085-1095.

82. Grosser,B.I.,Sweat,M.L.,Berliner,D.L.,Dougherty,T.F.(1962) Arch. Biochem. Biophys.96,259-264.

83. Iida,S.,Nakamura,Y.,Fujii,H.,Tsugawa,M.,Gomi,M.,Tarui,S.,Moriwaki, K.,Kitani,T.(1989) 71st Endocrine Society Meeting Abstract #644

84. Iida,S.,Nakamura,Y.,Fujii,H.,Nishimura,J.I.,Tsugawa,M.,Gomi,M., Fukata,J.,Tarui ,S.,Moriwaki,K., and Kitani,T.(1990) J. Clin. Endocrinol. Metab. 70, 729-737.



FOR REFERENCE

NOT TO BE TAKEN FROM THE ROOM

BRO DART CAT. NO. 23 012 PRINTED IN U.S.A.