Synthesis of 18-Hydroxycortisol and 18-Oxocortisol in Bovine Adrenal Zona Glomerulosa Mitochondria (43605)

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Abstract. **The biosynthesis of 18-hydroxycortisol and 18-oxocortisol from cortisol was studied in calf adrenal zona glomerulosa mitochondria. Cortisol is converted to 18-hydroxycortisol and 18-oxocortisol in the same mitochondrial preparation in which corticosterone is metabolized to 18-hydroxycorticosterone and aldosterone. Cortisol and 18-hydroxycortisol interacted with mitochondria to cause a Type I differential spectrum, which was decreased by sodium dithionite. The metabolism of cortisol to 18-hydroxycortisol and 18-oxocortisol was inhibited by metyrapone in a competitive way.**

Cortisol was a competitive inhibitor of the transformation of corticosterone into 18-hydroxycorticosterone and aldosterone, and corticosterone was a competitive inhibitor of the transformation of cortisol into 18-hydroxycortisol and 18-oxocortisol, with a K_i very similar to the K_m for the transformation of that steroid to aldosterone. These results indicate that cortisol is metabolized to 18-hydroxycortisol and 18-oxocortisol by a mitochondrial cytochrome P-450, which is the same as that which catalyzes the conversion of corticosterone into aldosterone. [P.S.E.B.M. 1993, Vol 203]

The isolation and identification of 18-hydroxycortisol as a quantitatively important steroid excreted in the urine of patients with primary aldosteronism were reported by Chu and Ulick (1). Cortisol incubated with the interrenal organ of the bullfrog, a model of zona glomerulosa, is hydroxylated to 18-hydroxycortisol and 18-oxocortisol, presumably following a pathway similar to that followed by corticosterone in its conversion to 18-hydroxycorticosterone (18-OH-B) and aldosterone (2). 18-Hydroxycortisol and 18-oxocortisol are secreted in excess in patients with primary aldosteronism due to adrenal adenomas and in glucocorticoid-suppressible aldosteronism (1, 3, 4). 18-Oxocortisol is a weak mineralo- and glucocorticoid, with a relative potency 1% that of aldosterone and 3% that of cortisol (5), respectively; it can raise the blood pressure when given chronically to rats (6) or sheep (7). 18-Hydroxycortisol is inactive as a mineralo- and glucocorticoid (8).

Studies of binding of steroids to adrenal mitochondria indicate that both corticosterone and cortisol bind specifically to the same protein, which might be a cytochrome P-450, suggesting that corticosterone metabolism and cortisol metabolism occur through the same enzymatic pathways (9).

The human, mouse, and rat adrenals have two cytochrome P-450 enzymes with 11β-hydroxylase activity (10–15), each of which has 18-hydroxylase activity. One enzyme is involved in the 18-hydroxylation of deoxycorticosterone to 18-hydroxydeoxycorticosterone (16–18), and a second hydroxylates corticosterone to 18-hydroxycorticosterone and aldosterone (18). Recent studies suggest that in the bovine, these two 18-hydroxylases might be only one enzyme. Two different cytochrome P-450 11β/18,19/-hydroxylase clones have been isolated, and both have aldosterone synthase activity (19–21). Outer bovine adrenal slices synthesize not only aldosterone, but also 18-oxocortisol. Inner adrenal slices do not synthesize either aldosterone or 18-oxocortisol (22). No clear explanation exists to account for the exclusive biosynthesis of aldosterone in the zona glomerulosa of the bovine adrenal if the same

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cytochrome P-450 11β-hydroxylase is present throughout the adrenal. The adrenal synthesis of 18-hydroxycortisol and 18-oxocortisol has not been well studied, but COS cells transiently transfected with the human aldosterone synthase cDNA not only convert deoxycorticosterone to 18-hydroxycorticosterone and aldosterone, but also convert 11-deoxycortisol to 18-hydroxycortisol and 18-oxocortisol, suggesting that the same enzyme is responsible for both biosynthetic conversions *in vivo* (15).

We report studies on the synthesis of 18-hydroxycortisol and 18-oxocortisol from cortisol by bovine zona glomerulosa mitochondria.

Materials and Methods

Materials. Most steroids and reagents for buffers were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents for high performance liquid chromatography were freshly distilled from reagent grade solvents. [1,2-^{3}H]Corticosterone and [1,2-^{3}H]cortisol were obtained from Amersham Corp. (Arlington Heights, IL). 18-Hydroxycortisol, 18-oxocortisol, [1,2-^{3}H]18-hydroxycortisol (sp act, 58 Ci/m*M*), and [1,2-^{3}H]18-oxocortisol (sp act, 50 Ci/m*M*) were synthesized as previously described (3, 23, 24). Aldosterone was measured by direct radioimmunoassay using a highly specific monoclonal antibody (25). 18-Hydroxycortisol, 18-oxocortisol, and 18-hydroxycorticosterone were measured by the combination of high performance liquid chromatography/radioimmunoassay as described previously (3, 22). Protein was determined by the method of Lowry *et al.* (26). Differential spectra were measured using a dual beam Aminco DW-2 spectrophotometer.

Preparation of Tissue and Subcellular Fractions. Calf adrenals were obtained from a local abatoir, placed in Hanks' balanced salt solution, and transported to the laboratory. The adrenals were trimmed free of fat and surrounding tissues, and slices of approximately 500 μm were cut using a Stadie-Riggs Microtome. The slices were homogenized in 0.25 *M* sucrose-20 m*M* HEPES buffer, pH 7.0, and subjected to differential centrifugation. In short, the homogenate was centrifuged at 600*g* for 10 min. The supernatant was then centrifuged at 11,000*g* for 20 min. The pellet was resuspended and centrifuged again. The mitochondria were resuspended in incubation buffer (20 m*M* HEPES, 210 m*M* mannitol, 70 m*M* sucrose, 5 m*M* magnesium chloride, and 5 m*M* calcium chloride, pH 7.1) to a final concentration of approximately 0.8 mg of protein/ml.

Incubations. Cortisol and other steroids were dissolved in 10 μl of propylene glycol and placed into 12 × 75-mm glass tubes. Mitochondria were added in 0.9 ml of incubation buffer and kept in ice for 30 min to allow penetration of the steroids inside the mitochondria. After adding 10 m*M* malate and 0.5 m*M* $NADP^+$ in 100 μl, the tubes were placed in a shaking incubator at 37°C for 8 min in air. The incubations were stopped by placing the tubes in ice and centrifuging at 5000*g* for 30 min. The supernatant was then separated and used for the measurement of 18-hydroxycortisol and 18-oxocortisol (3, 22, 24).

The formation of aldosterone and 18-hydroxycorticosterone was studied by incubating mitochondria, as described above, with [1,2-^{3}H]corticosterone of various specific activities. After incubating as described above, aldosterone was measured by direct radioimmunoassay (25), and 18-hydroxycorticosterone was measured by high perfomance liquid chromatography/radioimmunoassay (22).

Differential Spectrum. Differential spectra were obtained from mitochondria suspended in 10 m*M* phosphate buffer, pH 7.4, supplemented with 10 m*M* sodium malate, 1 m*M* $NADP^+$, and 0.1 m*M* potassium cyanide. The incubation contained 1.5 mg of mitochondrial protein in 2 ml. After obtaining the baseline, 2 μM steroid was added in 5 μl of dimethylformamide. The reference cuvette received an equal amount of the solvent.

Results

Time Course. The formation of 18-hydroxycortisol and 18-oxocortisol from 200 μM cortisol and that of 18-hydroxycorticosterone and aldosterone from 200 μM corticosterone were linear up to 10 min, as shown in Figure 1. An incubation time of 8 min was chosen for all subsequent incubations.

Cofactor Requirements. When mitochondria were incubated with 30 μM cortisol for 8 min in the presence of 0.5 m*M* NADPH, $NADP^+$, NAD^+, or NADH, maximal generation of 18-hydroxycortisol and 18-oxocortisol was obtained in the presence of NADPH. All subsequent studies were performed with a NADPH-generating system (malate and $NADP^+$; see Materials and Methods).

Differential Spectrum. Incubation of mitochondria with cortisol and 18-hydroxycortisol produced a Type I differential spectrum, with a trough at 418 nm and a peak at 390 nm. Differences in absorbance between peak and trough were 0.122 and 0.084 absorbance units (AU) for cortisol and 18-hydroxycortisol, respectively. These differences were diminished to 0.027 and 0.06 AU, respectively, by the addition of sodium dithionite, and further increased to 0.086 and 0.047 AU by subsequent oxygenation.

Determination of K_m and V_{max}. The K_m and V_{max} for the transformation of corticosterone into 18-hydroxycorticosterone and aldosterone were 12.3 μmol and 0.7 μmol/min. mg of protein, and 3.7 μmol and 0.2 μmol/min/mg of protein, respectively.

The K_m and V_{max} for the conversion of cortisol into 18-hydroxycortisol and 18-oxocortisol were 12.8 μmol

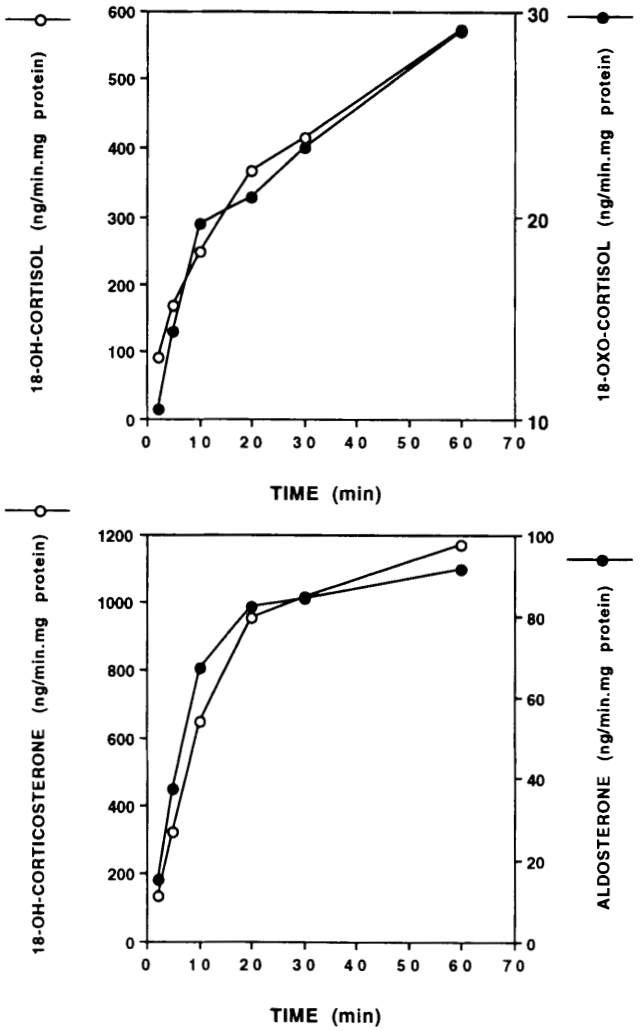

Figure 1. Time course for the formation of 18-hydroxycortisol (18-OH-CORTISOL) and 18-oxocortisol incubated with cortisol (200 μM) and of 18-hydroxycorticosterone (18-OH-CORTICOSTERONE) and aldosterone incubated with corticosterone (200 μM). Data are the mean of triplicate determinations from two incubations.

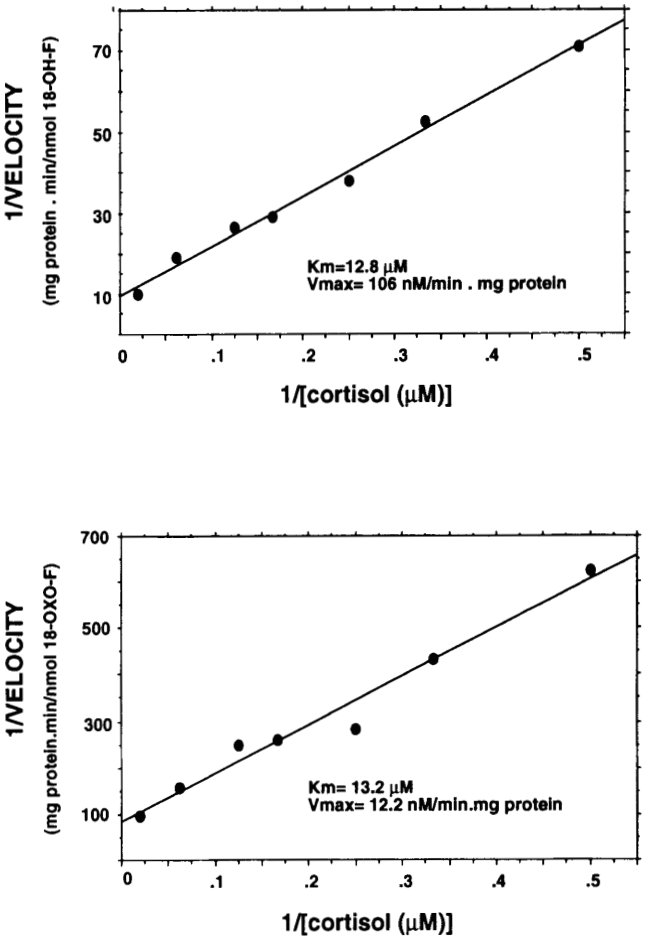

Figure 2. Lineaweaver-Burk plots for the transformation of cortisol into 18-hydroxycortisol (18-OH-F) and 18-oxocortisol (18-OXO-F). Mitochondria were incubated for 8 min in the presence of 15 m*M* malate, 0.5 m*M* NADP, and increasing concentrations of cortisol.

and 106 nmol/min/mg of protein and 13.2 μmol and 12.2 nmol/min/mg of protein, respectively (Fig. 2).

Conversion of 18-Hydroxycortisol to 18-Oxocortisol. Cortisol and 18-hydroxycortisol (30 μM) were incubated with mitochondria, as described above, for 8 min; 18-oxocortisol generation was 10.8 ± 0.42 (±SD) and 1.22 ± 0.11 pmol/mg protein/min (n = 3), respectively.

Inhibition Experiments. Metyrapone inhibited the formation of 18-hydroxycortisol (K_i = 86.65 nM) and 18-oxocortisol (K_i = 53.01 nM) from 10 μM cortisol (Fig. 3).

Cortisol at a constant concentration was incubated together with corticosterone at various concentrations, and the formations of 18-hydroxycortisol (K_i = 4.35 μM) and 18-oxocortisol (K_i = 4.11 μM) were measured. Dixon plots indicate that the inhibition was competitive (Fig. 4).

In a similar fashion, a constant amount of corticosterone was incubated with various concentrations of cortisol, and the formations of 18-hydroxycorticosterone (K_i = 12.2 μM) and aldosterone (K_i = 13.4 μM) were determined. Dixon plots indicated competitive inhibition.

Discussion

Cortisol was metabolized by calf adrenal mitochondria and converted into 18-hydroxycortisol and 18-oxocortisol by the cytochrome P-450 11β,18-hydroxylase. This conclusion is supported by the following findings. Both cortisol and 18-hydroxycortisol generated a Type I differential spectrum when interacting with mitochondria, wich was inhibited by the addition of sodium dithionite, and the conversion of cortisol to 18-hydroxycortisol and 18-oxocortisol was inhibited by metyrapone, a well known inhibitor of cytochrome P-450 11β-hydroxylase. The low difference in absorbance for 18-hydroxycortisol compared to cortisol is in agreement with the much lower conversion of the first steroid to 18-oxocortisol (data not shown). This situation re-

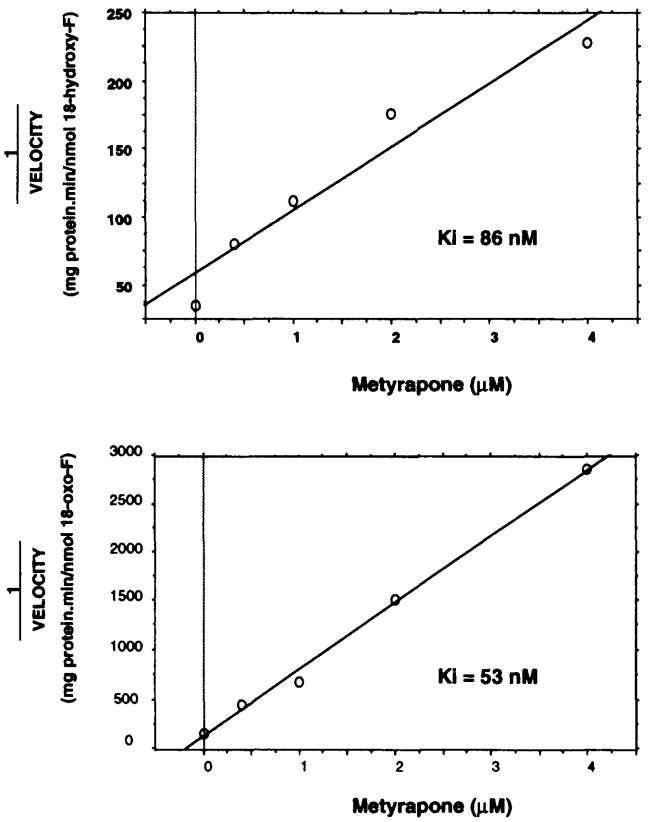

Figure 3. Dixon plot for the inhibition by metyrapone of the transformation of cortisol into 18-hydroxycortisol (18-hydroxy-F) and 18-oxocortisol (18-oxo-F).

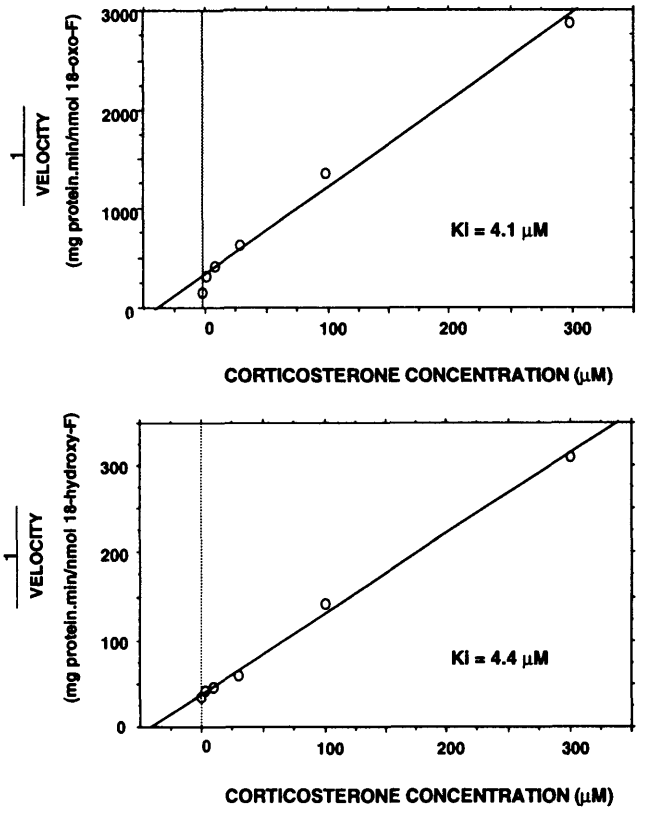

Figure 4. Dixon plots for the inhibition by corticosterone of the transformation of cortisol into 18-hydroxycortisol (18-hydroxy-F) and 18-oxocortisol (18-oxo-F).

sembles the difference in between the conversion of corticosterone and that of 18-hydroxycorticosterone to aldosterone (27).

The cytochrome P-450 involved in the metabolism of cortisol is the same as that involved in the metabolism of corticosterone to produce 18-hydroxycorticosterone and aldosterone. The inhibition of cortisol metabolism by corticosterone and the inhibition of corticosterone metabolism by cortisol indicated that only one enzyme, namely the cytochrome P-450 11β-hydroxylase (aldosterone synthase), catalyzed the conversion. These results are in agreement with our previous findings of steroids binding to mitochondria (9). The fact that the K_i values for both inhibitions were similar to the respective K_m values supports the concept that both corticosterone and cortisol are acting as competitive inhibitors.

Rats, mice, and humans have two distinct cytochrome P-450 11β-hydroxylases, and one of them has aldosterone synthase activity (10–15). In the bovine and porcine species, there appears to be a single enzyme that functions only as a 11β-hydroxylase in the zona fasciculata and as both 11β-hydroxylase and aldosterone synthase in the zona glomerulosa (20, 28, 29). We have previously reported that 18-ethynyl-deoxycorticosterone inhibits aldosterone production without affecting cortisol biosynthesis, suggesting that the metabolism of deoxycorticosterone to aldosterone in calf adrenals is very similar to that in species where two enzymes have been described (30).

The synthesis of aldosterone occurs in the zona glomerulosa and not in the zona fasciculata (31, 32), whereas cortisol is synthesized in the zona fasciculata and not in the zona glomerulosa, which allows the adrenal cortex to function as two distinct systems with different functions and regulations. 11β-Hydroxylase from bovine or porcine mitochondria copurifies with the "corticosterone methyl oxidase" activity, and immunoprecipitation with antibodies against 11β-hydroxylase coprecipitate the corticosterone methyl oxidase, suggesting that both are the same enzyme (19, 20). The lack of synthesis of aldosterone in the zona fasciculata may be due to either the way the enzyme is inserted into the inner mitochondrial membrane or the presence of an inhibitor of aldosterone synthesis in zona fasciculata mitochondria (32, 33).

Adrenal cells originate in the subcapsular area of the adrenal and lack 17-hydroxylase (34, 35). The steroids synthesized are 17-deoxysteroids, such as corticosterone, 18-hydroxycorticosterone, and aldosterone. During maturation, the cells migrate centripetally, and 17-hydroxylase is induced under the influence of

ACTH (35). The "aldosterone synthase" activity diminishes as the cells migrate centripetally and cannot be detected in the zona fasciculata. We have postulated that in the interphase between the zona glomerulosa and zona fasciculata, or transitional zone, both enzymes coexist, leading to cortisol acting as a suboptimal substrate for the aldosterone synthase, resulting in the synthesis of 18-hydroxycortisol and 18-oxocortisol (36). Chronic ACTH administration to humans results in a marked increase in the synthesis of 18-hydroxycortisol and 18-oxocortisol (37). Aldosterone excretion in the urine reaches a maximum after 2 days and decreases to baseline levels by the fourth day of chronic ACTH administration (37). 18-Oxocortisol excretion reaches a maximum at 3 days and decreases slightly toward the fifth day of chronic ACTH administration, but excretion of the steroid is 26-fold greater than that of the control. ACTH induces 17-hydroxylase in the same cells that have aldosterone synthase; progesterone is then 17-hydroxylated to 17-hydroxyprogesterone and 21-hydroxylated to 11-deoxycortisol, which serves as substrate for the 11β/18-hydroxylase to give 18-oxocortisol as the main metabolic product.

In the disease state known as glucocorticoid-suppressible aldosteronism, there is a marked increase in the excretion of 18-hydroxycortisol and 18-oxocortisol (3) that results from an uneven crossover recombination of the 11β-hydroxylase and aldosterone synthase genes (38). The promoter region of the 11β-hydroxylase is incorporated to the coding region of aldosterone synthase, allowing the chimeric enzyme to be expressed in the zona fasciculata where the abundant suboptimal substrate cortisol is converted into 18-hydroxycortisol and 18-oxocortisol (38).

In conclusion, calf zona glomerulosa mitochondria contain an 11β-hydroxylase that converts corticosterone into 18-hydroxycorticosterone and aldosterone and can equally convert cortisol into 18-hydroxycortisol and 18-oxocortisol.

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