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EFFECTS OF PREGNANCY ON STEADY-STATE KINETICS OF 21-HYDROXYLASE AND 11β-HYDROXYLASE IN THE RAT ADRENAL GLAND

THOMAS F. OGLE
Department of Physiology, Medical College of Georgia, Augusta, GA 30902, U.S.A.

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SUMMARY

The effects of pregnancy on the steady-state kinetic constants of 21-hydroxylase and 11β-hydroxylase were studied in rat adrenal microsomes and mitochondria, respectively. Progesterone and 11-deoxycorticosterone (DOC) served as substrate for the respective assays. The apparent K_M values for 21-hydroxylase were lower on the mornings of proestrus (12 ± 1 μM), day 22 (D22) of pregnancy (10 ± 2 μM) and on the first day post-partum (12 ± 2 μM) than on the mornings of days 5 (D5) and 12 (D12) of pregnancy, 31 ± 6 and 28 ± 6 μM, respectively ($P < 0.01$). Enzyme activity was not lower at D5 or D12 than at proestrus or post-partum. V_{max} attained highest value at post-partum. No significant differences were observed in the apparent K_M for 11β-hydroxylase (1.9 to 5.4 μM). However, V_{max} was higher on proestrus and post-partum than during pregnancy ($P < 0.05$). S.A. of the enzyme was not altered, although total adrenal content of enzyme activity was greatest post-partum ($P < 0.01$). 21-Hydroxylase at D5 and D12 shows characteristics of mixed inhibition relative to the post-partum enzyme. On the other hand the proestrus and D22 enzyme exhibits characteristics of non-competitive inhibition relative to the post-partum enzyme. 11β-Hydroxylase exhibits characteristics of non-competitive inhibition at D5, D12, and D22 relative to the proestrus and post-partum enzyme. These findings suggest the presence of either a single effector which may change in concentration at D5 and D12 or multiple effectors which act simultaneously to decrease apparent affinity of enzymes for substrate and decrease V_{max}. These changes seem to account for the 80–85% decline in plasma corticosterone levels observed in resting rats during mid-pregnancy.

INTRODUCTION

Corticosterone plays an indispensable role in mammary development and initiation of lactation in the rat [1–3]. On the other hand, sufficiently high plasma levels of corticosterone can induce fetal death [4]. Perhaps as a consequence, resting levels of plasma corticosterone are maintained at much lower concentrations at days 5 and 12 of pregnancy than on day 22, post-partum, or during the estrous cycle. These changes result from diminished capacity of whole adrenal homogenates to convert progesterone to corticosterone [5]. The present study describes variations in kinetic characteristics of 21-hydroxylase and 11β-hydroxylase which seem to account for the observed decline in corticosterone production in pregnant rats. Just how these alterations might be synchronized with the concept that ACTH influences corticosterone production by controlling the rate-limiting step of cholesterol side-chain cleavage [6,7] is not yet known.

MATERIALS AND METHODS

Biological material. Long–Evans rats weighing 270 ± 9 g were maintained under a constant photoperiod of 12 h light: 12 h dark and a temperature of 22°. Pregnancy was determined by first appearance of vaginal sperm (Day 1 of pregnancy). Pregnant rats were killed on days 5 (D5), 12 (D12), 22 (D22) and within 24 h after parturition on day 23 (post-partum). The D22 group was killed 5–8 h prior to expected parturition. Proestrus rats were killed after exhibiting at least three consecutive 4-day cycles. All animals received food and water *ad libitum*. Animals were sacrificed by rapid decapitation between 0830 and 1000 h. Adrenal glands were quickly removed, cleaned of adhering fat, and homogenized in a solution of ice-cold 50 mM Tris–HCl, pH 7.4, and 0.25 M sucrose at 30 mg of adrenal tissue per ml.

Preparation of subcellular fractions. The homogenates were centrifuged at 900 *g* for 10 min to remove nuclei, unbroken cells, and red blood cells. The supernatant was then centrifuged at 9,500 *g* for 10 min. The supernatant was carefully removed and frozen for subsequent studies of 21-hydroxylase. The pellet (mitochondrial fraction) was resuspended in Tris–sucrose and spun again at 9,500 *g* for 10 min. The pellet, transferred to fresh Tris–sucrose, was resuspended at a concentration equivalent to 60 mg adrenal tissue per ml. This suspension of mitochondria was used in subsequent studies of the 11β-hydroxylase system. The microsome fraction containing the 21-hydroxylase system was prepared by spinning the supernatant of the 9,500 *g* spin at 25,000 *g* for 15 min, this supernatant was then subjected to an additional spin of 105,000 *g* for 90 min. The resultant microsomal pellet was resuspended in Tris–sucrose buffer at 60 mg adrenal tissue per ml. All preparative procedures were carried out at 4°. Protein concentration of each fraction was determined by the method of Lowry *et al.* [8].

Incubation conditions for studies of 21-hydroxylase. One hundred μl of the microsomal suspension (20–30 μg of protein) was added to 200 μl of incubation medium buffered at pH 7.4 with 50 mM Tris–HCl, containing 100 mM sucrose, 80 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 4.5 mM nicotinamide, 1% BSA (Fraction V, Sigma), and 1% glycerol. Reducing equivalents were provided by addition of an NADPH generating system (10 μm glucose-6-phosphate, 1 U glucose-6-phosphate dehydrogenase, E.C. 1.1.1.49, and 0.4 μm NADP per incubation tube). Preliminary studies showed that this system provides NADPH in excess. Substrate was a mixture of tritiated-progesterone (105 Ci/mM, New England Nuclear, Boston) and unlabelled progesterone (1,000–3,000 d.p.m./nmol). The labelled steroid was first purified on a Sephadex LH 20 column (16 cm) in a solvent system of benzene–methanol (85:15 v/v).

Initial velocity of 21-hydroxylation was determined by incubating the microsomal preparation with 3, 6, 10, 16, 24, and 30 μM of labelled progesterone substrate, delivered to each incubation tube in 5 μl of Tris–redistilled ethanol (1:1 v/v). The reaction was stopped after 1.5 min at 37° in air with 5 ml chloroform. 5,000 d.p.m. [^{14}C]-deoxycorticosterone was added to correct for procedural losses, and steroids extracted by shaking. The chloroform extract was dried, and taken up in 8 drops of benzene–methanol (85:15 v/v) and applied to 16 cm Sephadex LH-20 columns. The 11-deoxycorticosterone (DOC) fraction was collected and quantified by liquid scintillation spectrometry. The ratio of $^{3}H/^{14}C$ remained constant after the first chromatography. Product formation was calculated as nmol/min/mg protein from the radioactivity eluted as DOC from the columns, corrected for procedural losses and the specific activity of the substrate. The quantity of enzyme in microsomal preparations was estimated by incubating 50 μl of the suspension with excess progesterone (83 μM) in a total vol of 300 μl using the same reaction mixture and conditions for incubation. DOC formation was determined after 1.5, 3, and 6 min. No 11β-hydroxylase activity could be detected in the microsomal preparation (less than 2 pmol/min per 100 μl of microsomal pellet). Kinetic parameters and 21-hydroxylase activity measured in samples frozen for as long as one week were not different from those determined with fresh tissue.

Incubation conditions for studies of 11β-hydroxylase. Kinetics of the 11β-hydroxylase system were studied by incubating 100 μl of the mitochrondrial suspension (60–75 μg of protein) with 400 μl of the same incubation medium used for 21-hydroxylation with the following changes: nicotinamide was absent and reducing equivalents were provided by 16 mM isocitrate and 32 mM malate. Enzyme activity was greater in the presence of isocitrate + malate than with isocitrate alone. NADPH did not support 11β-hydroxylation unless Ca^{2+} was also present. These observations are in agreement with the findings of other workers [9] for rat adrenal 11β-hydroxylase and indicate that mitochondrial membranes were intact.

Five μl of DOC substrate dissolved in Tris–redistilled ethanol (1:1 v/v) was added to each incubation tube. Initial velocity was determined by incubating mitochondria with 2, 4, 6, 10, 12, and 16 μM of substrate for 1.5 min at 37° in air. The quantity of enzyme in mitochondrial preparations was estimated by saturating the enzyme (50 μl of mitochondrial suspension) with substrate (50 μM DOC) and incubating for 1.5, 3, and 6 min at 37°. The reaction was terminated by addition of 5 ml of freshly distilled chloroform and the corticosterone yield was determined by acid fluorescence [10].

Each of the four enzyme assays were carried out with adrenal tissue from individual animals. The velocity of reaction is expressed as nmol product formed/min/mg protein. Incubation of enzyme preparations without substrate and with substrate at zero time served as controls.

Determination of kinetic constants. A plot of the reciprocal of the initial velocity against the reciprocal of substrate concentration resulted in a straight line for each enzyme preparation. Initial velocity was constant at short time intervals; furthermore, measurements of initial velocity were made such that less than 10% of added substrate was converted into product. Under these conditions the Michaelis constant (K_M) represents the apparent dynamic dissociation constant in the steady-state. Thus K_M measures apparent affinity of enzyme for substrate under steady-state as opposed to equilibrium conditions [11]. Kinetic constants were estimated by computerized weighted regression analysis of double reciprocal plots of initial velocity vs. substrate concentration, and by the direct linear plot method of Eisenthal and Cornish-Bowden [12,13]. These methods gave similar mean values for apparent K_M and maximum velocity (V_{max}). The results of statistical analysis were essentially the same, although the direct plot method resulted in greater precision as has been observed previously [14]. The latter values are presented in this report. Statistical evaluation consisted of the Duncan multiple range test. Homogeneity of variance was evaluated by Bartlett's Test and appropriate transformations made when heterogeneity of variance was encountered [15].

RESULTS

Effect of protein concentration and time

Figure 1 illustrates the effects of microsomal and mitochondrial protein concentration and time on 21- and 11β-hydroxylation of progesterone and DOC, respectively, by rat adrenal glands. In each case, product formation is proportional up to a protein concentration of at least 35 μg/300 μl and 140 μg/500 μl for each preparation, respectively, under the standard conditions of assay ($r < 0.99$). Formation of product is a linear function of time for at least 6 min ($r < 0.99$). Rate of product formation exhibits the same degree of dependence on protein concentration

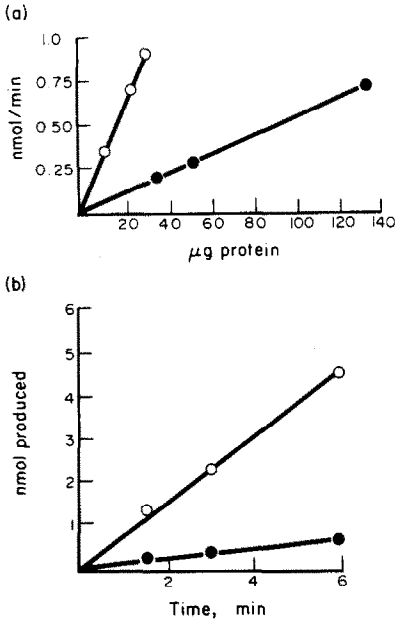

Fig. 1. The effect of protein concentration and time on 21- and 11β-hydroxylation by rat adrenal microsomes and mitochondria, respectively. (a) Enzyme activity is plotted versus amount of protein in each incubation tube. (b) Total product formation is plotted as a function of time. 21-Hydroxylation shown by open circles; 11β-Hydroxylation shown by closed circles.

and time for each reproductive condition. All experiments were performed under conditions of linearity with respect to protein concentration and time.

Studies of 21-hydroxylase in rat adrenal microsomes, Table 1

The amount of microsomal protein per 100 mg of adrenal tissue is low on proestrus and D5 but becomes markedly elevated thereafter ($P < 0.05$). Specific activity of 21-hydroxylase is highest on D5 ($P < 0.01$) and activity at proestrus is elevated above that measured at D22 and post-partum ($P < 0.05$). However, total adrenal content of 21-hydroxylase activity is highest at D5 of pregnancy ($P < 0.01$), but remains rather constant at other stages. Kinetic studies reveal that the apparent K_M for 21-hydroxylase is lower on proestrus, on the morning of parturition (D22), and on the first day post-partum than during D5 and 12 of pregnancy ($P < 0.01$). V_{max} is highest on D5 of pregnancy and post-partum. The mean values for each group indicate very complex kinetic alterations. On proestrus and D22 the enzyme exhibits characteristics of non-competitive inhibition relative to the post-partum enzyme (i.e., V_{max} decreased, apparent K_M unchanged). On the other hand the enzyme shows signs of mixed inhibition at D5 and 12 relative to post-partum (i.e., decreased V_{max} and increased apparent K_M).

Studies of 11β-hydroxylase in rat adrenal mitochondria, Table 2

The relative and absolute amount of mitochondrial protein declines from proestrus to their lowest values on D5. Mitochondrial protein increases to highest levels just prior to term (D22) and post-partum. The concentration of 11β-hydroxylase activity declines to a minimum at D22 ($P < 0.01$), but no other substantive changes occur. However, adrenal content of 11β-hydroxylase activity becomes enhanced post-partum ($P < 0.01$). Kinetic studies indicate that apparent K_M values for 11β-hydroxylase during proestrus and post-partum ranged between 35 and 64% of those during pregnancy. However, K_M values exhibited much more variation between individual rats during pregnancy than in non-pregnant rats. As a result the means were not significantly different. V_{max} was higher during proestrus and post-partum than in pregnancy ($P < 0.05$). These findings indicate that during pregnancy 11β-hydroxylase exhibits characteristics of non-competitive inhibition relative to the proestrus and post-partum enzyme.

DISCUSSION

This study was undertaken to examine kinetic characteristics of 21- and 11β-hydroxylase systems to better understand the mechanisms controlling adrenal function during pregnancy. It was previously noted that resting plasma levels of corticosterone decline by 80–85% from the proestrus level of 12 μg/100 ml to 2 and 3 μg/100 ml on days 5 and 12 of pregnancy,

Table 1. Effect of pregnancy on adrenal 21-hydroxylase

	Proestrus	Days pregnant 5	12	22	Post-partum
Microsomal protein (mg/100 mg ad. wt.)	0.43 ± 0.03 (14)§	0.40 ± 0.02 (10)‡	0.54 ± 0.04 (10)	0.51 ± 0.02 (12)	0.52 ± 0.02 (11)
(mg/ad. gl. pr.)	0.29 ± 0.03 (14)‖	0.22 ± 0.02 (10)*	0.35 ± 0.03 (10)	0.37 ± 0.02 (12)	0.36 ± 0.03 (11)
21-Hydroxylase activity (nmol deoxycorticosterone/min/mg protein)	32.0 ± 5.8 (6)‖	84.5 ± 5.9 (8)*	26.2 ± 6.1 (7)	14.7 ± 1.9 (6)	16.5 ± 1.2 (6)
(nmol deoxycorticosterone/min./ad. gl. pr.)	7.1 ± 1.5 (6)	21.0 ± 3.7 (8)*	9.6 ± 2.5 (7)	5.0 ± 1.0 (6)	7.0 ± 0.9 (6)
K_M (μM)	12.2 ± 1.5 (11)†	30.5 ± 6.4 (8)	28.2 ± 5.8 (10)	9.6 ± 2.1 (12)†	12.4 ± 1.8 (8)†
V_{max} (nmol deoxycorticosterone/min/mg protein)	19.4 ± 2.0 (11)	34.1 ± 4.0 (8)*	29.1 ± 3.7 (9)	22.5 ± 4.6 (12)	48.2 ± 5.6 (10)**

Values are means ± S.E. Number of animals in parentheses. * Value differs from all other stages, $P < 0.01$. † Value differs from D5 and D12, $P < 0.01$. ‡ Value differs from D12, D22, and Post-Partum, $P < 0.01$; § $P < 0.05$. ‖ Value differs from D22 and Post-partum, $P < 0.05$. ¶ Value differs from proestrus, $P < 0.05$. **Value differs from proestrus, D12, and D22, $P < 0.01$.

Table 2. Effect of pregnancy on adrenal 11β-hydroxylase

	Proestrus	Days pregnant 5	12	22	Post-partum
Mitochondrial protein (mg/100 mg ad. wt.)	1.07 ± 0.06 (12)§	0.97 ± 0.04 (12)‡	1.10 ± 0.02 (8)	1.28 ± 0.08 (12)	1.23 ± 0.07 (11)
(mg/ad. gl. pr.)	0.71 ± 0.06 (12)	0.50 ± 0.03 (12)*	0.69 ± 0.01 (8)*	0.94 ± 0.08 (12)	0.90 ± 0.09 (11)
11β-Hydroxylase activity (nmol corticosterone/min/mg protein)	2.2 ± 0.1 (6)	1.7 ± 0.2 (10)	1.9 ± 0.1 (7)	0.7 ± 0.1 (6)*	2.2 ± 0.4 (7)
(nmol corticosterone/min/ad. gl. pr.)	1.4 ± 0.1 (6)	0.9 ± 0.2 (10)	1.2 ± 0.1 (7)	0.8 ± 0.3 (6)	3.2 ± 1.1 (7)*
K_M (μM)	1.9 ± 0.3 (10)	5.4 ± 1.2 (9)	3.6 ± 0.7 (10)	4.9 ± 1.4 (12)	2.3 ± 0.3 (12)
V_{max} (nmol corticosterone/min/mg protein)	4.0 ± 0.4 (9)†	2.8 ± 0.3 (11)	2.8 ± 0.1 (8)	2.3 ± 0.4 (12)	3.7 ± 0.3 (12)†

Values are means ± S.E. Number of animals in parentheses. * Value differs from all other stages, $P < 0.01$. † Value differs from stages of pregnancy, $P < 0.05$. ‡ Value differs from D22 and Post-partum groups. $P < 0.01$. § Value differs from D22 group. $P < 0.05$.

respectively, which then increase to 10 μg/100 ml on day 22. Highest levels are observed on the first day post-partum (18 μg/100 ml). These alterations appeared to be determined by the capacity of whole adrenal homogenates to convert progesterone to corticosterone ($r^2 = 0.87$). Thus suggesting that perhaps changes in either 21-hydroxylase or 11β-hydroxylase may determine the rate of corticosterone production during pregnancy. Plasma levels of corticosterone from cycling rats showed no such dependence on these hydroxylating systems ($r^2 = 0.06$) [5]. In the present study, enzyme activity was not lower at D5 and D12 than other stages indicating that the observed differences in corticosterone production are not the result of a decline in amount of enzyme. However, kinetic parameters of both enzymes are altered on D5 and D12 relative to the enzymes assayed on the day of proestrus and post-partum. 21-Hydroxylation limits conversion of progesterone to DOC by having less apparent affinity for substrate at D5 and 12 (i.e., apparent K_M is higher). On the other hand, similar changes in apparent K_M of 11β-hydroxylase occurred during this interval, but could not be statistically established. However, V_{max} of 11β-hydroxylase declined during pregnancy ($P < 0.05$). Enzyme activity, the best estimate of enzyme concentration available for a crude preparation, was not altered. Thus, assuming a constant amount of enzyme, V_{max} may be diminished by an effector which alters the rate at which product is released from the catalytic site of the enzyme ($V_{max} = k_2 E_t$), i.e., the enzyme has a slower rate of turnover on D5 and D12.

These findings indicate very complex interactions between enzyme–substrate–effector. Relative to 21-hydroxylase at post-partum, the enzyme at proestrus and D22 shows characteristics of non-competitive inhibition. On the other hand, the enzyme at D5 and 12 shows mixed inhibition which may result from several effectors acting simultaneously or from changes in effector concentration [16]. 11β-Hydroxylase exhibits characteristics of non-competitive inhibition at D5, 12, and 22 relative to the proestrus and post-partum enzyme.

The adaptative significance of these kinetic changes during mid-pregnancy have not been determined. However, corticosterone production may be maintained at low levels to prevent premature lactation [17] or perhaps to allow normal fetal development [4]. The nature and regulation of the effectors responsible for the observed kinetic alterations and their dependence upon the hormones of pregnancy are presently under investigation.

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